

Studies on Drosophila ADH

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ABSTRACT

The polymorphism in the ADH locus of *Drosophila melanogaster* has been extensively investigated by many laboratories, particularly as a test system for probing the neutrality versus selection dilemma in evolution and population genetics.

In the present investigation, two rare ADH variants (not polymorphisms) ADH-F' and ADH-US, identified by David (1978), have been structurally studied. A high proportion (80%) of the amino acid sequence of ADH-F' has been detected. The only difference revealed between ADH-S and ADH-F' is the alanine - glutamic acid substitution at position 51. The extent of sequence information for ADH-US is limited, as this variant did not respond to the normal purification techniques employed and the very small amounts of pure enzyme obtained did not allow definitive sequence investigation. However, it has been established that it incorporates in its sequence the lysine-192 of the S allele.

A preliminary steady-state kinetic investigation of the pH dependency of the Michaelis constant for isopropanol of the ADH-F, ADH-S and ADH-F' enzymes and NAD^+ analog studies showed that ADH-F' is substantially similar kinetically to ADH-S. Thermal denaturation experiments also identified ADH-F' as an S-like variant.

The kinetic results showed that differences in properties exist between the major alleles (S and F). On the basis of these differences a model of balancing selection for the maintenance of polymorphism is suggested, that is in agreement with laboratory selection experiments and the geographical distribution of the major ADH allele frequencies.

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ABBREVIATIONS

Dansyl	1-dimethylamino naphthalene 5-sulphonyl
Tris	2-amino-2(hydroxymethyl)-1,3 propanediol
v/v	ratio by volume
w/v	ratio of weight to final volume

The three and one letter code for the common amino acids in proteins

Glycine	Gly, G
Alanine	Ala, A
Valine	Val, V
Leucine	Leu, L
Isoleucine	Ile, I
Serine	Ser, S
Threonine	Thr, T
Phenylalanine	Phe, F
Tyrosine	Tyr, Y
Tryptophan	Trp, W
Methionine	Met, M
Cysteine	Cys, C
Aspartic acid	Asp, D
Glutamic acid	Glu, E
Asparagine	Asn, N
Glutamine	Gln, Q
Histidine	His, H
Lysine	Lys, K
Arginine	Arg, R
Proline	Pro, P

CHAPTER 1 Introduction

1.1 Neutrality versus selection

1.1.1 Historical background of current theories

Differences in several traits among individuals of human, animal and plant populations have been recognised and recorded since antiquity, but their importance in natural history was neglected until Darwin's theory of evolution became the focus of controversy in the scientific world in the late 19th century. According to Darwin's hypothesis evolution is a gradual process, brought about by natural selection acting on these differences. The theory also presupposed that variation was inherited, but a mechanism of inheritance, fitting the theory, had to wait for the rediscovery of Mendel's laws and the revolutionary work of Morgan that proved them and linked the Mendelian "factors" or "genes" with the chromosomes.

With the advent of modern genetics that stemmed from Morgan's discoveries, it was realised that the number of these differences or *polymorphisms in a natural population is maintained and /

- * Ford (1940) defined as polymorphism the situation where members of a natural population can be categorised in two or more discontinuous forms, the rarest of which exists in frequencies that cannot be maintained by recurrent mutation.

and shaped by various processes. In synopsis, immigration from near-by populations and mutation tend to increase natural variation, while selection and genetic drift (accidental loss of variation) tend to control and limit it.

With these forces in mind, a number of theorists among whom Fisher, Haldane and Wright were the most prominent, settled down to investigate the mechanisms of natural selection in populations. A number of mathematical models were proposed explaining how mutation, drift and selection could account for the observed frequencies of polymorphic traits.

In part, today's debate is still an echo of the argument between Fisher and Wright about the assignation of drift as the dominant factor in controlling variation.

The study of the adaptive significance of polymorphisms was proven by the experimental work of Dobzhansky (1970), who demonstrated the influence of environmental factors on the frequencies of chromosomal polymorphisms. More importantly Dobzhansky's work showed that natural selection and adaptation could be studied effectively in the laboratory, under carefully controlled conditions.

Both theoretical and experimental work, aimed at examining the genetical make-up of populations, were thought to be complementary. But in the 1960's they became foci of two divergent schools of thought: the "neo-classicists" or "neutralists" and the "selectionists". The argument arose as certain features of the theoretical work were shown to be in need of readjustment since estimates about the /

the extent of polymorphisms were drastically altered.

When the theorists, in the 1930's, compiled their mathematical models, genetic variation was not thought to be widespread. Polymorphism was the exception, not the rule. Muller (1950), following experimental results on irradiated *Drosophila* populations, proposed that the majority of mutations are harmful. The reduction of fitness, due to mutant loci, was called the genetic load. Because of it, the amount of heterozygosity that a population could tolerate was severely limited. Other components of the genetic load were also postulated besides the mutational one described by Muller (1950). Since stabilising selection was known to act on some loci, several genotypes were less fit than others, being the cause of selective deaths. This was called the segregational load. Haldane (1957) calculated that a population, on the average, could cope with only one substitution per locus every 300 generations. Any more would lead to the extinction of the population. This burden that substitutional rates impose on the survival of populations was defined as the substitutional load. Kimura and Crow (1964) showed that, theoretically, the total genetic load becomes intolerably large if polymorphism is maintained in a significant number of loci by overdominance with appreciable selection coefficients.

1.1.2 Current theories based on evaluation of variation by electrophoresis

All the theoretical work that assumed a low amount of heterozygosity /

heterozygosity as a prerequisite for fitness was shaken seriously by the advent of electrophoresis as a diagnostic probe of genetic variation in natural populations.

Electrophoresis can distinguish substitutions in proteins if they result in changes of the net charge. Since on the simplest hypothesis one would predict that one third of all substitutions would be detected by this method, the technique should provide an objective estimate of polymorphism.

Harris (1966), surveying ten human enzymes, found three of them polymorphic. Lewontin and Hubby (1966), after studying 18 loci in *Drosophila pseudobscura*, estimated that the average population is polymorphic for about 30% of loci. According to the theoreticians, these populations ought to have died out because of their excessive genetic load. But this was clearly not the case.

Soon, theories, attempting to reconcile the new evidence with the previous theoretical work, were proposed by Kimura (1968 a, b) and King and Jukes (1969). Retaining the old estimates of the genetic load - which were challenged by a number of workers - they stated that the overwhelming majority of the mutations were either neutral or slightly deleterious. So, since selection is not acting upon them, the genetic load is not overlarge, and genetic drift is the major force shaping variation in natural populations. Thus, the obvious deduction from these theories is that if natural selection is insignificant in controlling variation then evolutionary change must be "non-Darwinian" (King and Jukes, 1969).

Such /

Such a drastic departure from accepted notions was not welcome to many investigators, who thereupon sought evidence to verify or falsify the predictions of the "neutralist" or "neo-classical" hypothesis.

Johnson (1974^b) showed differences in the amount of variation displayed by regulatory and non-regulatory enzymes, while Harris et al (1977) found a significantly lower incidence of polymorphism in multimeric than in monomeric enzymes. These differences, hinting at evolutionary pressures, disagreed with the neutralists assumptions of uniformity expected if variation is governed by random drift.

But population surveys and selection experiments proved more successful in upsetting the theorists. Powell (1971) showed that cages with relatively complex environments retain more heterozygosity in wild populations of *Drosophila* than cages with uniform environments. Ayala et al (1971), Ayala et al (1972), Prakash et al (1969), Ayala and Tracey (1974) and Ayala et al (1974), surveying natural populations of several *Drosophila* species, found that frequencies of various electrophoretic alleles tended to be constant. These authors considered that if random drift is the major force controlling variation the frequency of alleles in these populations ought to have diverged markedly.

The immediate response of the neutralist theoreticians was to assume that migration was responsible for the uniformity of electrophoretic frequencies. But since from the neutralist theory heterozygosity was assumed to be proportional to the population size, ($n_e = 1 + 4 N_e u^*$ according to Kimura and Crow, 1964) /

($n_e = 1 + 4 N_e u^*$ according to Kimura and Crow, 1964) the new, super-populations must have contained an incredibly large number of alleles, much higher than the one observed.

In order to resolve this difficulty, Kimura and Ohta (1973) attempted to put an upper limit to the heterozygosity expected. In the new model the selectively neutral mutations could affect the net charge of the protein by a unit of charge at most in either direction. The predicted number of neutral alleles was now proportional to the square root of the effective population size ($n_e = \sqrt{1 + 4 N_e u}$).

This modification of the neutralist theory did not restrain mounting criticism, revolving mainly around the connection between heterozygosity and population size, as Ayala and co-workers (1971/1974)** showed that in large populations there were stable equilibria of electrophoretic frequencies, independent of population size.

Thus attempts to put an upper limit to heterozygosity were abandoned. Instead, the principle of the distinguishability of alleles was attacked by the neutralists, on the basis of reports (Berstein et al, 1972) that electrophoresis had significantly underestimated the extent of polymorphism and that electrophoretic alleles (or electromorphs) were only phenotypes.

Ohta and Kimura (1975) demonstrated, theoretically, that the /

* N_e = effective population size

u = rate of mutation

** Mentioned in detail on page 5

the stable equilibria observed by Ayala et al (1971/1974) and discussed by Lewontin (1974) could arise if the fitness of random alleles is reduced by a small amount that is a function of the deviation from the optimal electrophoretic charge.

King and Ohta (1975), elaborating on this theoretical scheme, proposed a model which assumes that every mutation reduces the fitness of the allele by an amount, i.e. s (the selection coefficient). Thus alleles that have accumulated 0, 1, 2, ... , n mutations will have a selective fitness of 1, $1-s$, $1-2s$, ... , $1-ns$ respectively and mutants will therefore accumulate in large populations as long as s is lower than the mutation rate u . Each of these classes of alleles will be heterogeneous (apart from the zero mutations alleles) as regards the net charge displayed, but will have a defined distribution. At equilibrium, the most common electromorph will contain the type allele and other sequences as well, that include amino acid substitutions that do not involve charge changes and the even number of multiple changes that result in the original net charge. So, every electromorph will be sequentially heterogeneous, with heterozygosity increasing with large population numbers. Contrary to the Ohta and Kimura (1975) model, one of the major assumptions of this model is that there is no direct effect of electrophoretic charge on the selection coefficient. It also assumes that it describes equilibria expectations. The authors maintain that in their calculations the effects of overdominance, dominance, heterosis or frequency-dependent selection, although they are known to exist, were not taken into /

into account, but that these omissions do not invalidate the model.

1.1.3 Molecular evolution and the neutrality concept

One of the implications of the neutrality concept was that evolutionary change as a phenomenon was largely related to polymorphism. Therefore, intraspecific variation is a function of interspecific variation. With natural selection relegated to a very minor role, random genetic drift was the major force leading to the fixations of substitutions (the overwhelming majority of which was regarded as selectively neutral). Polymorphism was thus a contemporary restricted glimpse of a long process.

Neutralist theoreticians derived support from Margoliash's (1963) observations that the number of residue differences in sequences of cytochrome c between large taxonomic groups were very nearly constant. Since time elapsed from the original divergence is the only thing that large taxonomic groups have in common, then time must be the parameter that determines the number of mutations accumulating in structural genes. This reasoning led to the adoption of the concept of the "molecular clock" of evolution (Margoliash, 1963; Zuckerkandl and Pauling, 1962). The neutralists argument was further reinforced by the supposed lack in functional differentiation in cytochrome c in different eucaryotic species (Smith et al, 1973; Byers et al, 1971

Kimura (1968a) using the "constant" rates of protein evolution for cytochrome c and heamoglobin (Margoliash and Smith, 1965; Buettner - Janus and Hill, 1965), /

1965; Buettner - Janus and Hill, 1965), calculated that the substitutional rate per genome was at least a hundred-fold higher than the substitutional load permissible for a species as evaluated by Haldane (1957). If Haldane and other theoreticians estimates were to stand, then Kimura (1968a) maintained, evolution must surely be due to neutral mutations fixed by genetic drift.

Detailed investigation soon showed that evolutionary rates in cytochrome c, either in a single line of descent or in separate lines is not constant (Fitch and Markowitz, 1970; Langley and Fitch, 1973, 1974; Fitch and Langley, 1976), thus upsetting a pivotal point of the neutralist argument. Kimura and Ohta (1971) accepted this divergence of evolutionary rates but stated that the differences observed were restricted to parts of the molecules, subject to some selection pressures, and did not affect the hypothesis.

The neutrality concept was further undermined by Margoliash et al (1976) - whose initial assertions helped in launching it - who showed that functional differentiation in cytochrome c does exist and changes in amino acid sequence can be related to changes in properties correlated to the cytological environment of the molecule and its relationship with its oxidase. Evolution of haemoglobin function has also become accepted (Riggs, 1976).

Meanwhile, Clarke (1970) challenged a statement by King and Jukes (1969) that most amino acid substitutions fixed during protein evolution are neutral in selective value. He showed that the majority of substitutions are not chemically equivalent. Jukes and /

and King (1971) replied that Clarke's calculations were based on arbitrary assumptions and that the data were manipulated.

1.1.4 Neutrality versus selection and the Drosophila system


In the early 1970's with the neutrality-selection controversy becoming fiercer, data available on polymorphism were inadequate in providing an answer. Therefore several investigators (e.g. Clarke, 1975; Vigue and Johnson, 1973) suggested that the resolution of the problem was only possible through the exhaustive examination of present-day polymorphism. The employment of multi-dimensional approach was proposed which would combine environmental biochemical and physiological considerations with the careful monitoring of the total amount of variation. The system considered to be most suitable for such an approach was the alcohol dehydrogenase polymorphism in *Drosophila melanogaster*.

1.2 /

1.2 The alcohol dehydrogenase system in *Drosophila melanogaster*

1.2.1 General

Alcohol dehydrogenase (alcohol: NAD^+ oxidoreductase E.C.1.1.1.1) activity in *Drosophila melanogaster* was first reported by Johnson and Denniston (1964) and Grell et al (1965). The enzyme oxidises alcohols to their corresponding aldehydes or ketones using NAD^+ as a cofactor. It has a broad specificity, being active on a range of aliphatic, primary or secondary, and aromatic alcohols containing up to eight carbon atoms. However, it is totally inactive on methanol.

Natural populations of *Drosophila melanogaster* were found to be polymorphic for two alleles, segregating at the locus, identifiable by their differential mobility during electrophoresis (O'Brien & McIntyre, 1969; Grossman et al, 1970; Vique & Johnson, 1973; Pipkin et al, 1973; Briscoe et al, 1975). The alleles were designated as ADH fast and ADH slow on the basis of their electrophoretic behaviour ( in agar gels at pH 8.5). The terms ADH I and ADH II were also used (Pipkin et al, 1973) but they did not obtain universal currency. The initials F and S are also used for fast and slow respectively.

Grell et al (1965) located the ADH gene on the second chromosome with a map position of 50.1 cMg, and a cytological position somewhere between bands 34E₃ and 35D₁. This was later verified by Sampsell (1977) and McKay (1981), who mapped variants at the same locus. /

at the same locus.

The bulk of the enzyme, during the larval stage of development, seems to be situated in the fat body, where it is present at very high concentrations, and in the intestine. Some activity is contained in the Malpighian tubules and carcass. The same distribution of the enzyme is observed during adulthood, when some activity is also present in parts of the genital apparatus (Ursprung et al, 1970).

During ontogeny, changes of enzyme activity can be represented by the U-shaped curve typical of most metabolic enzymes. Activity is at a maximum in the 3rd instar larval stage and early after enclosion, and drops substantially during the pupation stage after enclosion (Ursprung et al, 1970; Ward, 1975; McDonald and Avice, 1976).

The role of the enzyme in the physiology of the organism remains obscure. Several roles have been proposed by different investigators:

- (a) The enzyme's primary function is detoxification and partial utilisation of the environmentally occurring ethanol (Gibson et al, 1970; Grell et al, 1968; McKenzie and Parsons, 1972).
- (b) The enzyme is involved in lipid metabolism (Johnson, 1974).
- (c) The enzyme has a role in temperature adaptation (Johnson and Powell, 1973).

The /

The function, that most investigators have assigned, centres around proposition (a) and the detoxification role of the enzyme was generally accepted. However, David and Bocquet (1976) noting the discrepancy between the in vitro behaviour of the enzyme (where it shows a marked preference for secondary alcohols) and the physiological observations (showing better survival for ADH⁽⁺⁾ organisms in ethanol containing media) remarked that the enzyme must derive from a so far undiscovered metabolic pathway and has only recently become employed in alcohol detoxification and utilisation.

1.2.2 Differences between the alleloenzymes

The two alleles (ADH-F and ADH-S) segregating in the ADH locus have been extensively investigated and the ADH-F allele was found to have higher specific activity than the ADH-S (Rasmusson et al, 1965; Gibson, 1970; Gibson and Miclovich, 1971; Vique and Johnson, 1973).

Gibson (1970) reported that the activity of the heterozygote was similar to the one displayed by F homozygotes, while Day et al (1974a) considered it to be intermediate between the two homozygotes.

Day et al (1974a) estimated that there was a lower K_m (app) for both cofactor and substrate in the S enzyme (results which were corroborated by McDonald et al, 1980) and considered that the discrepancies in activity were wholly accountable by significant differences /

differences in the catalytic efficiencies (k_{cat}) of the alleloenzymes.

Day et al (1974a) also demonstrated that the amount of enzyme produced by the two alleles is similar. Gibson's (1970) contradictory results were explained by the aforementioned authors as resulting from the less sensitive techniques employed.

Apart from specific activities, the two alleloenzymes were found to differ in heat stability, with the F the most sensitive of the two (Gibson, 1970; Vigue and Johnson, 1973; Day et al, 1974b; Sampsell, 1977; McKay, 1981). The stability displayed by the heterozygote was found to be intermediate by Gibson (1970), but Day et al (1974b) thought it to be very close to the one reported for the ADH-F homozygote.

Differences were also detected in the substrate specificities of the two variants by Day et al (1974b) and Chambers et al (1981). The F allele was shown to be only slightly more active on n-propanol than the ADH-S but twice as much on iso-propanol.

Although, broadly speaking, ADH-F/ADH-S flies display higher activity than either ADH-F/ADH-F or ADH-S/ADH-S flies throughout ontogeny extensive fluctuations in activity have been reported for these genotypes in individuals of natural populations and inbred lines (Birley & Barnes, 1973; Hewitt et al, 1974; Ward, 1974; 1975; Barnes & Birley, 1975; Kamping & van Delden, 1978; McDonald & Ayala, 1978).

Modifier genes accounting for these differences and supposedly acting directly on ADH were reported and mapped on the 2nd, 3rd and X chromosomes by Ward (1975), on the 3rd chromosome by McDonald and Ayala (1977), and on the X chromosome by Pipkin and Hewitt (1972), Hewitt (1974), and Birley and Barnes (1975).

Clarke et al (1979) cast doubts on the previously mentioned reports by presenting data that showed that the amount of ADH produced was "allosteric" with body weight, being proportional to the square of the body weight.

1.2.3 Selection as the cause of maintenance of polymorphism in the ADH locus of *Drosophila melanogaster*

Chambers et al (1979), surveying different oxidising enzymes in a number of *Drosophila* species, found that ADH activity exhibited the greatest amount of variation, which suggested either influence by environment factors or a role in adaptation. But earlier than this, a series of reported experiments and surveys has focused on the identification of these factors and their role in the maintenance of the ADH polymorphism.

(a) Ethanol and other alcohols as the selective factors

Because of the assumed function of the enzyme as the detoxifying agent of alcohol-rich foodstuffs (section 1.2.1) a number of selection experiments under laboratory controlled conditions have been conducted by several investigators to test this postulated mechanistic /

mechanistic connection between several alcohols and the ADH alleles products.

Gibson (1970) reported that the F allele frequency increased considerably in a 6% ethanol-supplemented medium. Similar increases were reported by Bijlsma-Meeles and van Delden (1974) in a 12% ethanol-supplemented medium, and by van Delden (1975), Cavener-Glegg (1978); Oakeshott (1976-1979).

The ADH-F/ADH-F genotype was found to confer higher fitness in ethanol-rich environments (Bijlsma-Meeles & van Delden, 1974; Morgan, 1975; Briscoe et al, 1975; Kamping & van Delden, 1978; van Delden & Kamping, 1979). On the other hand, Oakeshott (1976-1979) reported that some of his experiments showed that the heterozygote processed higher fitness than either homozygotes, but he agreed with van Delden and Kamping (1979) that in the presence of ethanol developmental times had positively regressed in the following order: ADH-F/ADH-F < ADH-F/ADH-S < ADH-S/ADH-S.

These discrepancies in results stem from the fact that selection and fitness experiments were performed in varying alcohol concentrations, and the way viability was tested differed with the investigator. (Eggs - to - adult, larvae - to - adult, or adults only).

The possibility that linkage disequilibrium might account for the results had been admitted by most workers. Van Delden et al (1978) checked their strains for chromosomal inversions, since linkage /

linkage disequilibrium had been reported by Mukai et al (1971) between ADH and several cosmopolitan inversions.

Also, in the experiments where ADH-F frequency was, at the start, over 0.5, the rise after 10 to 15 generations was marginal even in high ethanol concentration (15%) (van Delden et al, 1978; Oakeshott, 1979). Oakeshott repeatedly (1976, 1979, 1980) has maintained that the simple correlation between ADH activity and alcohol tolerance, assumed by several investigators, does not exist. For instance, maintenance of a population on a methanol-rich medium, which ADH cannot utilise, also leads to a significant increase of ADH-F frequency (Bijlsma-Meeles & van Delden, 1974). Another criticism of the laboratory selection experiments is that little account is taken of behavioural responses. No choice of environment is offered to the fly when such a choice is freely available in nature.

Several mechanisms for the maintenance of the ADH polymorphism have been proposed that are derived from the fitness and selection experiments that are described above. Kojima and Tobari (1969) suggested a frequency dependent mode of selection on the basis of experimental results that they presented. The frequency dependent mode of selection originally postulated by Haldane has the significant advantage that it reconciles theoretical estimates of segregational load with selection acting on the locus. In equilibrium selection will not be acting and therefore the genetic load of such polymorphism will be zero. Kojima and Yarrow (1967) claimed /

claimed to have also detected frequency-dependent selection on the esterase locus of *Drosophila*. Oakeshott (1976) thought that his results showed heterozygote advantage as the force maintaining the ADH polymorphism but van Delden and Kamping (1978) objected claiming that overdominance was the only mechanism able to explain the experimental observations. Clarke and Allendorf (1980) thought that preliminary kinetic results on the ADH polymorphism agreed with the frequency-dependent mode of selection proposed by Kojima and Tobari (1969) if the fly utilised ethanol directly as a food source. But Dolan and Robertson's (1975) results failed to show any conditioning of the food by the *Drosophila* larvae as regards ethanol, a prerequisite for frequency-dependent selection. The latter authors also thought that Oakeshott's postulated mechanism was not supported by the bulk of the evidence.

Contradictory results were also reported from population surveys. While Briscoe et al (1975) reported that in Spanish wine cellars ADH-F was virtually monomorphic, McKenzie (1974); McKenzie & McKechnie (1978) showed that although alcohol tolerance was significantly increased in the vicinity of wine cellars in several Australian vineyards, the ADH alleles frequency remained unaltered. Gibson (1979) obtained similar results to the latter group.

David and Bocquet (1977) presented results showing alcohol tolerance to be a quantitative character (a multi-genic one). Alcohol tolerance progressively increased during directional selection. The same authors are in agreement with Gibson (1979) regarding /

regarding ADH only as part of this complex trait in *Drosophila*. Gibson (1979) further maintained that ADH can contribute to the alcohol tolerance mechanism only when the 'alcohol shock' exceeds certain values. He also remarked that the different results obtained by Briscoe et al (1975) might be due to the higher alcohol content of the Spanish cellars investigated and by the fact that ADH-S is held in linkage disequilibrium by the common cosmopolitan inversion In(2L)t, whereas no such linkage was displayed by the Australian populations.

Although the effects of environmental alcohols on the ADH polymorphism are debatable, there seems to be firm evidence that the increased activity of ADH in *Drosophila melanogaster* (in comparison with other species of this genus) is favouring its colonisation of alcohol-rich environments. McKenzie and Parsons (1972) reported that *Drosophila melanogaster* was almost unchallenged in the fermentation cellars, while *Drosophila simulans* was prevalent in adjacent alcohol-free areas. Laboratory selection experiments by David and Boquet (1976) confirmed that *Drosophila simulans* is very sensitive to high alcohol concentrations, its behaviour approaching that displayed by ADH (null) individuals of *Drosophila melanogaster*. McDonald and Avise (1976) have shown that there is a positive correlation between the higher ADH content of *Drosophila melanogaster* and its survival in ethanol-rich environments compared with nine other *Drosophila* species.

(b) Temperature as the selective factor /

(b) Temperature as the selective factor

As discussed above, another difference between the two alleloenzymes is heat stability, with the S one the most resistant to heat denaturation. Johnson and Powell (1974) subjected laboratory populations to extremely severe heat-and-cold shocks and found that heat shocks select against the S allele while the cold ones have the opposite effect. Bijlsma-Melles and van Delden (1974) also showed that the ADH-S extinction rate is much higher than the ADH-F one at low temperatures.

These laboratory results were supported by Vigue and Johnson (1973), who showed a clinal distribution of ADH allele frequencies, with ADH-S dropping from 0.9 in the North American South to 0.5 in the North. It must be noted that ADH-S has been reported linked to the In(2L)t inversion which shows a similar clinal distribution, falling from 0.25 in the North American South to about zero in the North (Vockler et al, 1978). Pipkin et al (1973), surveying populations for *Drosophila melanogaster* in Southern Texas and Mexico, found that populations in areas of high mean temperature are almost monomorphic for the S allele. But . . . calculations failed to show any correlation between ADH allele frequencies and extreme maximum temperatures. Surprisingly, a significant positive correlation was revealed between extreme minimum and mean temperatures and the regression of the frequency of the ADH-S allele. Wilks et al (1980) reported similar results based on frequency distribution of the two alleles in Australian populations of *Drosophila melanogaster*. /

populations of *Drosophila melanogaster*.

Although these results are consistent with their being a role for the enzyme polymorphism in adaptation strategies to temperature fluctuations, the mechanistic relationship remains obscure whereas with alcohols as selective factors a mechanistic relationship can easily be postulated.

1.3 /

1.3 The alcohol dehydrogenase gene and gene product

1.3.1 The isoenzymes

Electrophoresis of *Drosophila melanogaster* ADH reveals that the enzyme exists in vivo in distinct forms that are distinguished by their different mobilities in an electric field. These forms, termed isoenzymes, are a common phenomenon displayed by several enzymes, e.g. mammalian ADH, LDH, *Drosophila* esterase in various organisms (Branden et al, 1975; Holbrook et al, 1975). The importance and the role of these "isoenzymes" or "chemical forms" are still elusive. They were investigated in *Drosophila* ADH because the difference in properties among them further complicated an already complex problem, that is the evolutionary and adaptational pressures on this locus.

In their initial description of the ADH system in *Drosophila melanogaster* using agar-gel electrophoresis, Johnson and Denniston (1964) reported the existence of only two forms, one of which - later termed ADH-3 - was rather faint. Most of the activity was concentrated in the cathodal band.

Grell et al (1965) and Ursprung and Leone (1965) using a more sensitive technique, detected three (3) bands of activity in the homozygotes. There was disagreement about the number of bands produced by the heterozygote, with Grell et al distinguishing nine (9) and Ursprung and Leone seven (7). It was /

was soon shown that two of the anodal bands in Grell's gels corresponded with another enzyme which was active on the same substrate, an octanol dehydrogenase (ODH) (Courtright et al, 1966). The bands displayed by the heterozygote included the parental set as well as the expected intermediates.

The whole picture was further complicated when Ursprung & Carlin (1968) and Jacobson (1968) observed that these intermediate bands could also be detected in homozygotes in the purified enzyme and in crude extracts treated with various chemicals such as β -mercapto-ethanol.

The different bands were designated by numbers, the counting starting from the most anodal form (Ursprung and Leone, 1965; Ursprung and Carlin, 1968) and the isoenzymes of the two alleloenzymes were assigned different numbers. This confusing system was soon abandoned when the alleloenzymes were removed from the isoenzyme nomenclature system. Jacobson (1968), Jacobson et al (1970); Jacobson et al (1972); Knopp et al (1972); Day et al (1974a, b) and Day and Needham (1974) assigned the number 5 to the most cathodal form of the two alleloenzymes. Number 1 was assigned to the most anodal form with numbers 2 to 4 attached to the intermediates (Fig. 1.1).

The isoenzymes were found to differ, sometimes quite markedly, in other properties apart from electrophoretic mobility. Differences in thermostability were noticed quite early by several /

several investigators (Grell et al, (1968); Gibson, (1970); Day and Needham, (1974); Schwartz et al, (1975)). It appears that the most cathodic form (ADH-5) is the least stable, with stability increasing to the more anodal forms. Although there is a significant difference in thermostability between the alleloenzymes as discussed previously, this gradient in the isoenzymes is evident in both F and S.

More differences were reported by Day and Needham (1974), who found that there is a marked difference in the temperature optimum in the isoenzymes, with ADH-S-5 achieving maximal activity at 37°C and ADH-S-3 at 50°C. The same authors managed to isolate partially these forms by electrofocusing. They showed that the specific activity of ADH-S-5 was twice as high as the one displayed by ADH-S-3 and that there is a significant discrepancy between these forms in the estimates for the true Michaelis - Menten constant for ethanol K_m^{eth} (app).

There was an extensive search for the factors responsible for the generation of the isoenzymes and their possible physiological and adaptive significance.

The original suggestion by Ursprung and Leone (1965) that the bands were due to different sub-units was soon rejected by Grell et al (1968) who observed that in ADH-D, and alleloenzyme artificially induced by ethyl-methanesulphonate mutagenesis, a single point mutation shifts the whole isoenzyme pattern towards /

towards the cathode. The obvious conclusion from such a result was that a common polypeptide chain was shared by all three forms. This would have been impossible if two different subunits existed. The belief in the existence of a single polypeptide chain was further reinforced with data from complementation experiments (Grell et al, 1968; Schwartz et al, 1975). The point was finally proven when the enzyme was purified by Sofer and Ursprung (1968); Jacobson et al (1970) and Thatcher (1977).

That the forms were not simply aggregates of the single polypeptide chain was shown by Imberski et al (1968), who observed that isoenzymes migrate at identical rates in different polyacrylamide concentrations and by Sofer and Ursprung (1968) and Jacobson et al (1970), who demonstrated identical sedimentation rates for all isoenzymes.

Amino acid analysis of different forms as well as peptide finger-printing, performed by Schwartz et al (1975), did not reveal any differences in the primary sequence of the various forms. Day and Needham (1974) and Anderson and McDonald (1981) found that the isoenzymes are immunologically identical.

Since it has been convincingly proven that the isoenzymes were not genetically determined, emphasis was shifted in investigating the effects of chemical modifications and conformational changes. Although most of the experiments were conducted in vitro, and they are subject to the usual scepticism of /

of such results, they did provide an insight into the isoenzyme generation.

Following reports by McKinley-McKee and Moss (1965) that NAD^+ alters the isoenzyme pattern of the mammalian ADH, Ursprung and Carlin (1968) observed that NAD^+ has the same effects on *Drosophila* ADH. But β -mercapto-ethanol was shown to be able to achieve this shift of staining intensity as well, Jacobson (1968) and Jacobson et al (1970) reported that the isoenzymes were interconvertible. ADH-3 passing through DEAE-cellulose columns was converted to ADH-5 although it must be noted that Sofer and Ursprung (1968) (who used DEAE-cellulose for their purification of ADH) observed no such conversion. It was further claimed by the former authors that excess NAD^+ in gels could transform ADH-5 to ADH-3 and ADH-1 during electrophoresis. The converted bands obtain the expected properties as far as specific and thermostability are concerned and correspond well with the forms observed in fly extracts. Jacobson (1968) eluted the thus generated ADH-3 and ADH-1 and showed them to possess more bound NAD^+ than ADH-5, from which they were derived.

Schwartz et al (1975) proposed that the generation of the bands was due to the binding of a negatively charged carbonyl- NAD^+ compound (reported to be bound but rather loosely on other dehydrogenases). Labelled nicotinamide was introduced to the fly food and, although the label was shown to remain on the nicotinamide, it was released in the supernatant after heat denaturation of the pure protein. /

protein. A substance is also released during denaturation that can, after incubation, transform ADH-5 to ADH-3. Some studies on this substance, when isolated, showed it to exhibit the properties expected of the NAD^+ - carbonyl compound originally postulated (Schwartz et al, 1975).

Subsequently, Schwartz and Sofer (1976), testing their hypothesis further, fed to flies compounds that are known to produce addition complexes with NAD^+ (i.e. 3-hydroxybutan-2-ol or acetoin). Flies thus fed showed a definite change in the isoenzyme patterns after 24 hours and the extent of the change depended on the amount of the substance in the food. Compounds that are either lethal when fed to the flies, or are known to be non-reactive towards NAD^+ did not produce a similar change. Anderson and McDonald (1980) reported similar results using isopropanol. From this a model was developed (Schwartz and Sofer, 1976) in which ADH-5 contains no bound NAD^+ complex but ADH-3 and ADH-1 have one and two molecules of this compound respectively.

However, Jacobson et al (1972) showed that the amount of bound NAD^+ found on ADH-1 varies without any consequences to the electrophoretic mobility. Furthermore, he showed that the rate of conversion of forms depends on NAD^+ and enzyme concentrations in such a way as to suggest that an impurity in NAD^+ is responsible for the conversion. Since acetone is a compound that usually contaminates NAD^+ preparations, acetone conversion was tried with positive results. Jacobson et al (1972) therefore concluded that conformational /

conformational changes were involved during conversion. This conclusion is in accordance with the results obtained by Lutstorf and von Wartburg (1968), who demonstrated that isoenzyme pattern conversion in the mammalian ADH is independent of NAD^+ .

Following this suggestion Knopp and Jacobson (1972) reported that fluorescence emission spectrum of the enzyme undergoes a shift to shorter wavelength during conversion from ADH-5 to ADH-1. The rate at which the shift is accomplished follows the rate of the decrease of activity where the decrease in relative quantum yield follows the extent of the conversion. According to the authors, these results positively associate conformational changes with isoenzyme conversion. The results also indicate that the conformational changes affect the environment of some, if not all, tryptophan residues.

Since only guesses can be made about the role of the ADH system in the physiology of the fly, it follows that speculation about the metabolic importance and adaptive significance of its isoenzymes are even more hazardous.

However, the temptation was not resisted by Jacobson et al (1970) who suggested a probable role in development, following reports from Dunn et al (1969) that ADH-2 is present only at certain stages of development. Day and Needham (1974) proposed an adaptive role for the thermostability differences observed, but Schwartz et al (1975) thought that the isoenzymes simply represented enzyme on its way to degradation.

In consideration of Heed's (1978) report that isopropanol is a major component of the alcohol content of the *Drosophila* environment, Anderson and McDonald (1981) speculated that the decrease of specific activity for ADH-3 and ADH-1 could be an adaptive response inhibiting the enzyme from converting isopropanol to its corresponding ketone, a far more toxic substance.

1.3.2 Molecular weight and sub-unit number

The enzyme has been purified to apparent homogeneity by a number of workers, using the conventional techniques of ammonium sulphate precipitation, hydroxyapatite chromatography, DEAE- or CM-cellulose chromatography, gel filtration and ultracentrifugation. Successful purifications have been reported by Sofer and Ursprung (1968); Jacobson and Pfuderer (1970); Schwartz et al (1975) and Thatcher (1977).

Leigh-Brown and Lee (1979) purified the enzyme using general ligand affinity chromatography, employing 8-hexyl-6-(amino)- α -AMP as ligand.

The molecular weight of the intact enzyme was estimated at 4.4×10^4 by Sofer and Ursprung (1968), using sucrose density centrifugation and gel filtration. This estimate was slightly adjusted by Schwartz et al (1975) after re-appraisal of the partial specific volume estimate used by Sofer, with amino acid analysis results.

A different, significantly higher estimate, was published by Jacobson and Pfuderer (1970). Using sedimentation equilibrium centrifugation and gel filtration, they calculated a molecular weight of 6×10^4 .

Although genetic analysis, and the number of hybrid bands present in heterozygotes in the ADH locus, defined the molecule as a dimer of two identical sub-units, Jacobson and Pfuderer (1970) reported the enzyme to be an octamer, consisting of identical sub-units, with a molecular weight of 7,500, calculated from SDS gels. Later Jacobson et al (1970), on the basis of amino acid analysis, reported a molecular weight of 15,000 Dlt for the sub-unit, reducing the number of sub-units to four.

Schwartz et al (1975) and Thatcher (1977), using the same techniques, estimated in SDS gels that the molecular weight of the sub-unit approximated 25,000, thus verifying the genetic results. Both these authors reported the existence in their preparations of small amounts of a contaminant protease, which could probably account for the low estimates published by Jacobson and Pfuderer (1970) and Jacobson et al (1970).

Accurate values are now available from the amino acid sequence of the protein which agree very well with the SDS gel estimates. The molecular weight for the sub-unit is 27,586 and for the dimer 55,172. These values include the small correction necessary to allow for the extra tryptophan residue revealed by the gene sequence (Benyajati et al, 1981). /

gene sequence (Benyajati et al, 1981).

1.3.3 Metal-ion content

The mammalian and yeast alcohol dehydrogenases depend on a zinc ion or a zinc-bound water molecule for activity. In direct contrast the alcohol dehydrogenase in *Drosophila* does not require metal ions for enzymatic activity. As Jacobson (1968) observed, metal chelators (i.e. EDTA, EGTA) fail to inactivate the enzyme. Place et al (1980), using X-ray fluorescence analysis, revealed only 0.02g-atoms of zinc per mole enzyme. Other metals were found in even smaller quantities.

But pyrazole, a specific inhibitor of alcohol dehydrogenases in yeast and mammals, does inactivate *Drosophila* ADH, as reported by Leigh-Brown and Lee (1979) and Place et al (1980). It is well established that pyrazole achieves inactivation by binding with the zinc ion, in the mammalian and yeast enzymes, of the active site. The reaction in the *Drosophila* enzyme must be different. Place et al (1980) observed that the enzyme-pyrazole-NAD⁺ complex in *Drosophila* ADH has a different ultraviolet absorption spectrum from the one recorded for similar complexes in the zinc alcohol dehydrogenases.

1.3.4 Isoelectric points

The published estimates for the isoelectric points of the
two /

two alleloenzymes disagree, but this disagreement probably reflects the techniques employed. Day et al (1974a), using a modified version of the Bloemental and Schoemaker (1968) method, estimated a value of about 8.05 for ADH-S-5 and 7.2 for ADH-F-5 at 4°C. There was a significant decline in the PI of the fast enzyme at 0.5°C, while temperature variations did not affect the S enzymes. Thatcher (1980) employed isoelectric focusing in thin-layer agarose gels, a technique which gave a value of 6.4 for the F-5 band and 7.0 for the S-5 one, at 4°C.

1.3.5 Enzyme kinetic studies of the Drosophila ADH alleloenzymes

Further differences between the Drosophila ADH and the equivalent mammalian and yeast enzymes were revealed by a detailed investigation of their substrate specificities. In Drosophila ADH there is a marked preference for secondary and cyclic alcohols (Sofer and Ursprung, 1968; Day et al, 1974a; Thatcher, 1977). In contrast the mammalian and yeast enzymes show a preference for primary alcohols (Dalziel, 1975).

Sofer and Ursprung (1968) observed that at high concentrations and for some alcohols (cyclohexanol, isopropanol, isobutanol) substrate activation is evident, a phenomenon observed by Dalziel and Dickinson (1966) for horse liver ADH and to explain which an enzyme - NADH - secondary alcohol ternary complex which dissociated faster than the enzyme - NADH - ketone was proposed.

Day /

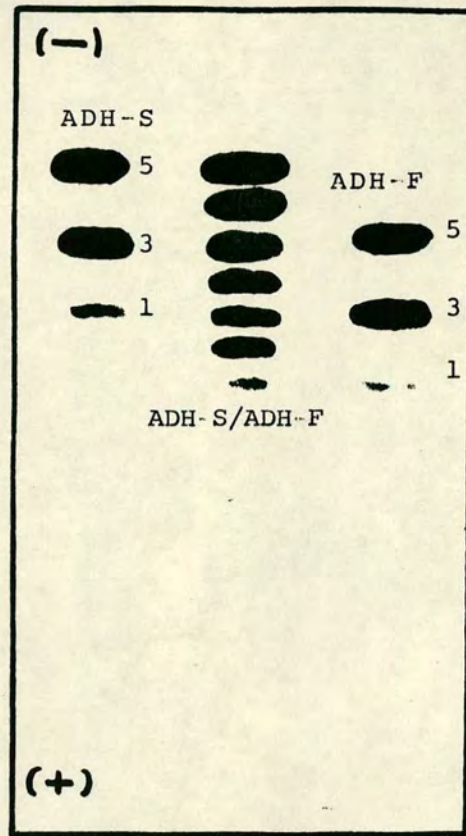


Fig. 1.1 : agar gel electrophoresis pattern of ADH alleloenzymes (F & S).

From Ursprung and Leone (1965)
(Isoenzyme designation is the currently employed one)

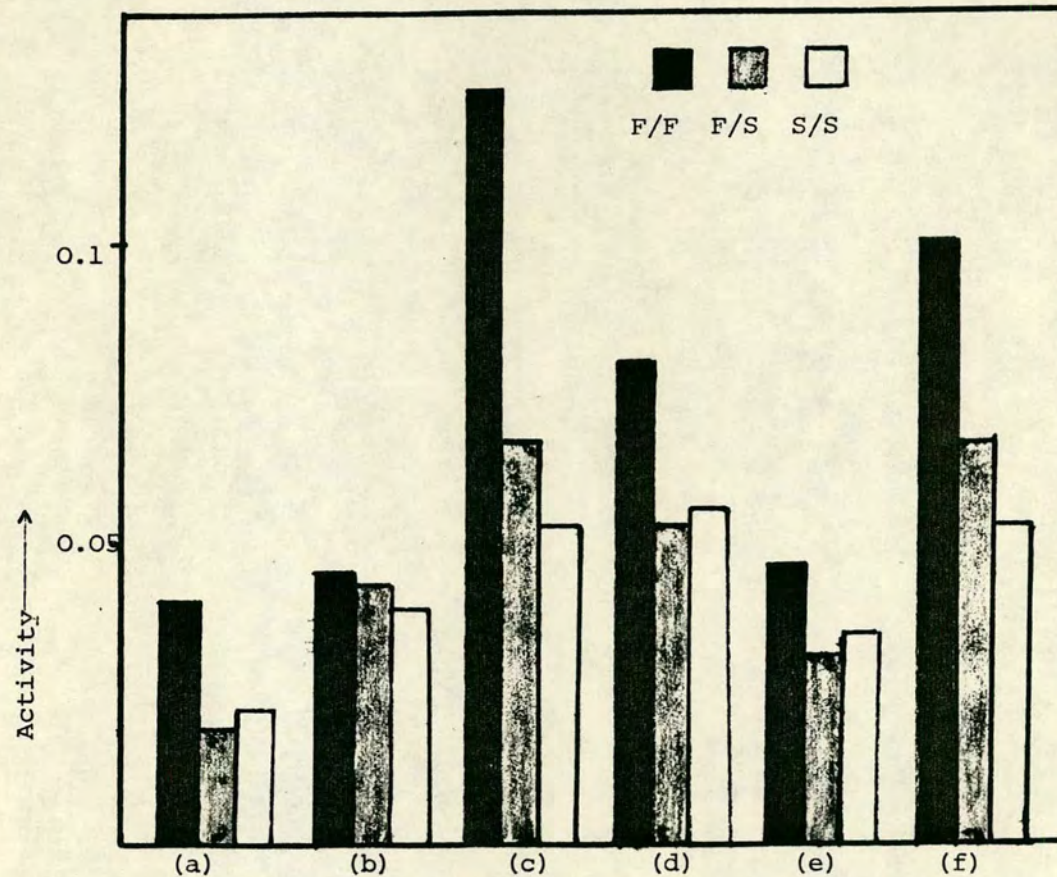


Fig. 1.2 : ADH activities of crude F/F, F/S and S/S extracts with: (a) Ethanol, (b) n-Propanol, (c) Isopropanol (d) n-Butanol, (e) Isobutanol, (f) Cyclohexanol.

From Day et al (1974a)

Day et al (1974a) found some differences between the substrate profile of the two alleloenzymes. The reactivity of the F variant is reduced significantly when isopropanol is replaced by n-butanol but an increase in reactivity is observed for the S. Also, while both alleloenzymes display almost similar activities when n-propanol is the substrate, the F is at least twice as active as the S when isopropanol or cyclohexanol are employed as substrate (Fig. 1.2).

Specific activities for both the alleloenzymes have been calculated in several experiments, but at different degrees of purity of preparations, at different pH values and at different ionic strengths. In most experiments the preparations contained the full complement of isoenzymes, which as discussed above possess different kinetic properties. Day et al (1974b) experimented with the most cathodal and the most active isoenzyme (band 5) of both alleloenzymes and established immunologically that the F ADH-5 had twice the specific activity of the S one, results that agree with the activity measurements on fly extracts mentioned in section 1.2.2.

Michaelis-Menten constants have been estimated for both F and S ADH-5 by Day et al (1974a). McDonald et al (1980) repeated these experiments with multibanded enzyme preparations, while the previous group's experiment normally employed only one isoenzyme (but the preparation was only partially pure). Estimation of these kinetic constants was done by both groups at a single pH value (different between groups) but there was agreement that the ADH-S has /

has a lower Michaelis-Menten constant for the cofactor (NAD^+).

McDonald et al (1980) reported also that the S enzyme exhibits 'positive' interaction for both substrate and cofactor (as NAD^+ concentrations increase K-alc decreases and as alcohol concentration increases K- NAD^+ decreases), while ADH-F exhibits 'negative' interaction (as concentrations of NAD^+ and alcohol increases K-alc and K- NAD^+ increase correspondingly). In contrast Day et al (1974b) observed positive interactions for both alleloenzymes. McDonald et al (1980) accounted for this disagreement by the different purity of their preparations.

1.3.6 Conformational differences between the alleloenzymes as revealed by heat denaturation experiments

Since temperature is one of the most important environmental factors, the thermostability of the alleloenzymes is of primary importance. But heat stabilities might also correlate with other properties, i.e. resistance to proteolytic degradation or other disruptive factors.

Day et al (1974a) while discussing the effects of temperature on the isoelectric point of ADH-F (section 1.3.4) suggested that a conformational change must occur in the F alleloenzyme at higher temperatures while ADH-S stays in the same conformation over the whole temperature range.

Initial /

Initial velocities studies by Thatcher and Sheikh (1981) showed that ADH-F-5 and ADH-S-5 differ only in their temperature optima, while exhibiting very similar temperature profiles. These temperature profiles are in good agreement with those published by Day et al (1974a), although the latter did not detect differences in temperature optima.

Irreversible denaturation by heating at pH 6.0 again revealed very small differences between the most cathodal isoenzymes of both alleloenzymes. When the heating rate is fast no differences in "melting" temperature can be detected but at a lower heating rate ADH-F-5 starts melting at a lower temperature than the ADH-S-5 enzyme (Thatcher and Sheikh, 1981).

However, heat denaturation experiments in a transverse temperature gradient during polyacrylamide gel electrophoresis - a technique developed by Thatcher and Hodson (1981) - showed a significant difference between the two alleloenzymes, with the melting point for ADH-F-5 at $32 \pm 0.9^{\circ}\text{C}$ and for ADH-S-5 at $38 \pm 1.0^{\circ}\text{C}$ (Thatcher and Sheikh, 1981).

At lower pH's (=4) sub-unit dissociation and reassociation was observed, and in such pH values the temperature gradient gels technique showed that the hybrid molecule (ADH-F-5/ADH-S-5) was as stable as ADH-S-5 (Thatcher and Sheikh, 1981).

Although most of the experiments performed demonstrated that ADH-F is less stable than ADH-S, they also showed that the convincingness /

reliability of such results depends critically on the experimental conditions employed. Great caution must therefore be applied to the extrapolation of such results to in vivo conditions.

1.3.7 Amino acid analysis, protein sequence and gene sequence

The amino acid analysis reported by Jacobson et al (1970); Schwartz et al (1975) and Thatcher (1977) showed that the enzyme did not contain any methionine residues. Two cysteine residues are present but no disulphide bridge is formed as demonstrated by the titration of a thiol group with 5,5' -dithiobis-(2-nitro-benzoic acid) (Thatcher, 1977). There was a disagreement between Thatcher (1977) and Schwartz et al (1975) about the number of tryptophan residues, which was not settled until the publication of the gene sequence that showed the existence of five, not four, such residues (Benyajati et al, 1981).

While tryptic digestion and peptide finger-printing showed at least 24 tryptic peptides - two more than expected - (Schwartz et al, 1975) not all of them could be obtained pure from peptide maps. Schwartz and Jörnval (1976) managed to obtain a partial sequence for 40% of the ADH-F sub-unit from these peptides. With this partial sequence of the F enzyme available, a similar survey of tryptic peptides for ADH-S by Fletcher et al (1978) revealed only one difference detectable in this part of the sequence. A tryptic peptide with a sequence Thr-Thr-Leu-Val-His-Lys was present in the ADH-S /

ADH-S tryptic digest, but was absent from the ADH-F one.

A homologous sequence in an ADH-F peptide of poor solubility

Thr-Thr-Leu-Val-His-Thr-Phe-Asp-Ser-Leu-Leu-Asp-Val-Glu-Pro-Gln-Val-

-Ala-Glu-Lys, led to the firm conclusion that the lysine residue of

the extra S peptide has been substituted by threonine in the F-enzyme.

The C-terminal sequence of the enzyme was established by Thatcher (1977) by carboxypeptidase -A digestion. No free N-terminus could be detected by end-group analysis, but mass spectroscopy proved that the enzyme possessed a naturally occurring acetyl group blocking the N-terminus, and also determined the two first amino acid residues of the N-terminal sequence (Auffret et al, 1978).

The protein was difficult to sequence, as the blocked N-terminus reduced the potential of automatic sequence analysis and from enzyme digests many peptides were of poor solubility. Nevertheless the full sequence of the enzyme was established and published by Thatcher (1980) and confirmed that the single Thr/Lys substitution between ADH-F and ADH-S was located at position 192.

This sequence was further confirmed by the sequencing of the DNA of the gene (Benyajati et al, 1981). Only two discrepancies were noticed. At position 25 the DNA sequence indicates the presence of glutamic acid residue instead of the glutamine (though Thatcher (1980) noted anomalous behaviour of this residue) while an additional tryptophan residue was detected in position 251. Two introns /

ATG TCG TTT ACT TTG ACC AAC AAG AAC GTG ATT TTC GTT GCC GGT CTG GGA GCC ATT GGT CTG GAC ACC AGC AAG CAG CTG CTC AAG CGC
 Ser-Phe-Thr-Leu-Thr-Asn-Lys-Asn-Val-Ile-Phe-Val-Ala-Gly-Leu-Gly-Gly-Ile-Gly-Leu-Asp-Thr-Gar-Lys-Glu-Leu-Leu-Lys-Arg
 1 10 20

GAT CTG AAG AAC CTG GTG ATC CTC GAC CGC ATT GAG AAC CCG GCT GCC ATT GCC GAG CTG AAG GCA ATC AAT CCA AAG GTG ACC CTC ACC
 Asp-Leu-Lys-Asn-Leu-Val-Ile-Leu-Asp-Arg-Ile-Glu-Asn-Pro-Ala-Ala-Ile-Ala-Glu-Leu-Lys-Ala-Ile-Asn-Pro-Lys-Val-Thr-Val-Thr
 30 40 50

TTC TAC CCC TAT GAT GTG ACC GTG CCC ATT GCC GAG ACC ACC AAG CTG CTG AAG ACC ATC TTC GCC CAG CTG AAG ACC CTC GAT CTC CTG
 Phe-Tyr-Pro-Tyr-Asp-Val-Thr-Val-Pro-Ile-Ala-Glu-Thr-Thr-Lys-Leu-Leu-Lys-Thr-Ile-Phe-Ala-Glu-Leu-Lys-Thr-Val-Asp-Val-Leu
 60 70 80

ATC AAC GGA GGT GGT ATC CTG GAC GAT CAG CAG ATC GAG CGC ACC ATT GCC CTC AAC TAC ACT GCC CTG CTC AAC ACC ACC ACC GCC ATT
 Ile-Asn-Gly-Ala-Gly-Ile-Leu-Asp-Asp-His-Glu-Ile-Glu-Arg-Thr-Ile-Ala-Val-Asn-Tyr-Thr-Gly-Leu-Val-Asn-Thr-Thr-Thr-Ala-Ile
 90 100 110

CTG GAC TTC TGG GAC AAG CGC AAG GCC GGT CCC GGT GGT ATC ATC TGC AAC ATT GGA TCC CTC ACT GGA TTC AAT GCC ATC TAC CAG GTG
 Leu-Asp-Phe-Trp-Asp-Lys-Arg-Lys-Gly-Gly-Pro-Gly-Gly-Ile-Ile-Cys-Asn-Ile-Glu-Ser-Val-Thr-Gly-Phe-Asn-Ala-Ile-Tyr-Glu-Val
 120 130 140

CCC GTC TAC TCC GGC ACC AAG GCC GCC GTG CTC AAC TTC ACC ACC TCC CTG GCG AAA CTG GCC CCC ATT ACC GCC CTG ACC GCT TAC ACC
 Pro-Val-Tyr-Ser-Gly-Thr-Lys-Ala-Ala-Val-Val-Asn-Phe-Thr-Ser-Ser-Leu-Ala-Lys-Leu-Ala-Pro-Ile-Thr-Gly-Val-Thr-Ala-Tyr-Thr
 150 160 170

GTG AAC CCC GGC ATC ACC CGC ACC ACC CTG GTG CAC ^{CTA} TTC AAC TCC TGG TTG GAT GTT GAG CCC CAG GTT GCT ^C GAG AAG CTC CTG GCT
 Val-Asn-Pro-Gly-Ile-Thr-Arg-Thr-Thr-Leu-Val-His-Lys-Phe-Asn-Ser-Trp-Leu-Asp-Val-Glu-Pro-Glu-Val-Ala-Glu-Lys-Leu-Leu-Ala
 180 190 200

CAT CCC ACC CAG CCA TCG TTG GCC TGC GCC GAG AAC TTC GTC AAG GCT ATC GAA CTG AAC CAG AAC GGA GCC ATC TCG AAA CTG GAC CTG
 His-Pro-Thr-Glu-Pro-Ser-Leu-Ala-Cys-Ala-Glu-Asn-Phe-Val-Lys-Ala-Ile-Glu-Leu-Asn-Glu-Asn-Gly-Ala-Ile-Trp-Lys-Leu-Asp-Leu
 210 220 230

GCC ACC CTG GAG GCC ATC CAG TCG ACC AAG CAC TGG GAC TCC GCC ATC **TAA**
 Gly-Thr-Leu-Glu-Ala-Ile-Glu-Trp-Thr-Lys-His-Trp-Asp-Ser-Gly-Ile
 240 250

Fig. 1.3 : Amino-acid and gene sequence of *Drosophila melanogaster* ADH-S. (arrows indicate the position of introns detected in the DNA sequence)

(from Benyazati et al, 1981)

introns were also detected. The smaller, 65 bases long, interrupts the sequence at position 32 and the longer, 70 bases long, at position 167 (Benyajati et al, 1981). Both DNA and protein sequence are shown in Fig. 1.3.

1.3.8 Secondary structure predictions

All the dehydrogenases studied so far contain two domains: the NAD^+ -binding domain and the catalytic domain. The NAD^+ -binding domains of alcohol dehydrogenases for which the 3-D structure is known through X-ray crystallography studies, show remarkable similarities in the secondary structure organisation, although there is little primary structure homology (Rossmann et al, 1975). This organisation consists of alternating β -pleated sheets and α -helixes with β - α - β as the basic unit.

Thatcher and Sawyer (1980) and Benyajati et al (1981) have published secondary structure predictions employing the Chou and Fasman (1974) method (Fig. 1.4). Although differences exist between the two predictions β - α - β pattern is evident in the N-terminal part of the enzyme. When aligned with the horse liver ADH, which structure is known there are significant similarities in secondary structure between the known and the presumed domains. In contrast to the *Drosophila* enzyme both liver ADH from mammalian sources and the yeast ADH have the NAD^+ domain in the C-terminal part of the molecule.

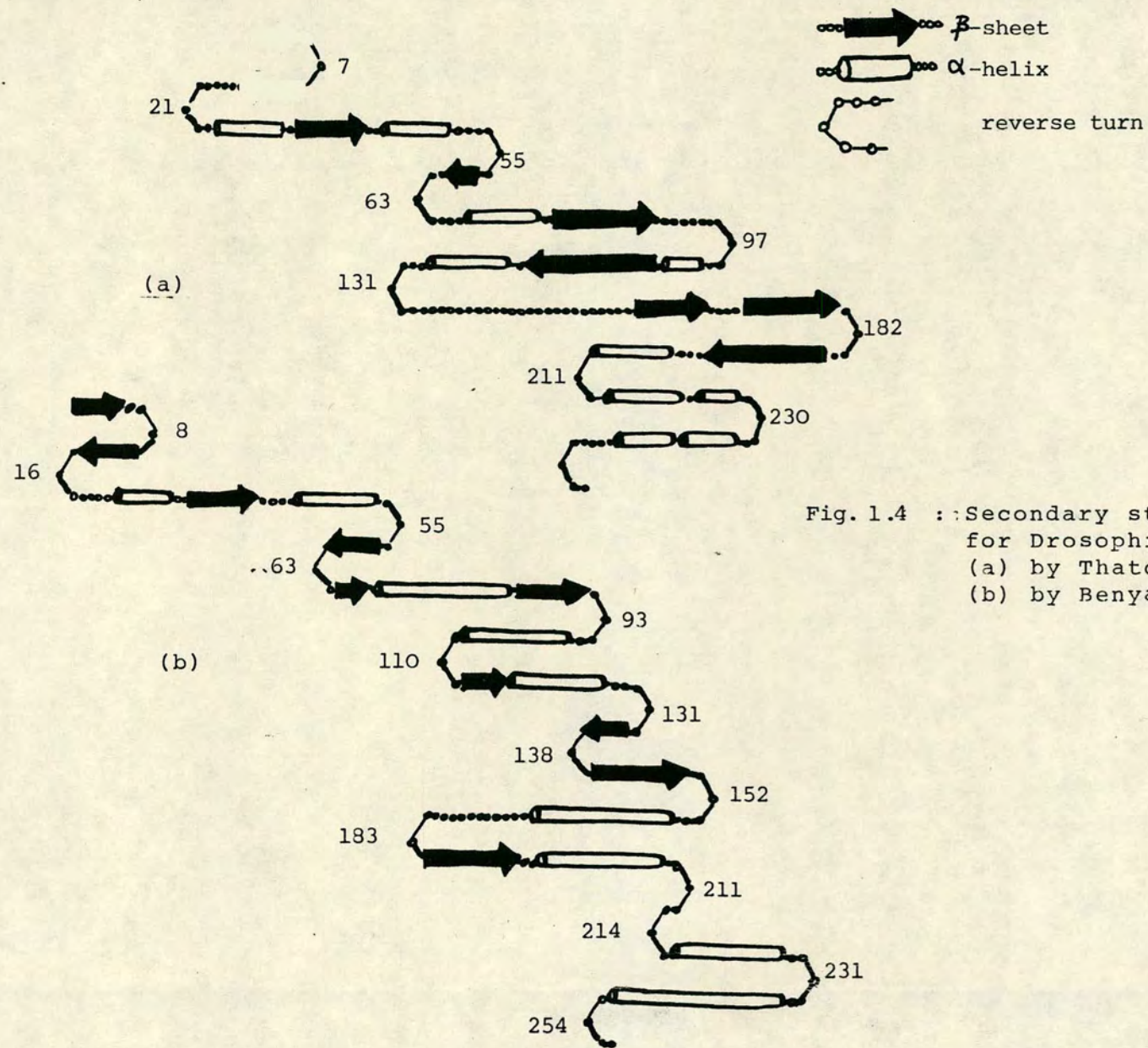


Fig. 1.4 :: Secondary structure predictions
 for *Drosophila* ADH:
 (a) by Thatcher and Sawyer (1980)
 (b) by Benyajati et al (1981)

Benyajati et al (1981) speculated on the function of the introns discovered in the sequence of the gene and noted that the longer one is found in position 167, in the boundary between the coenzyme binding and catalytic domains. However no special function can yet be seen for the first 32 residues of the N-terminal sequence, which the smaller intron separates from the rest of the sequence. Benyajati et al (1981) commented that this N-terminal segment showed a definite resemblance to the secondary structure of a synthetic 34 residue polypeptide, designed by Gutte et al (1979), to bind polynucleotides.

1.4 The present investigation

The present investigation has mainly been concerned with two recently discovered *Drosophila melanogaster* ADH variants. The ADH-F' (F prime) enzyme, to which a substantial part of this thesis is devoted, is a naturally occurring variant that differs slightly in electrophoretic mobility from the F alleloenzyme (David, 1978). ADH-US (ultra slow) - partially investigated here - differs in charge from the ADH-S by a unit (David, 1978). Neither of these variants qualify as polymorphisms by the conventional definition as their frequencies in all populations investigated have always been below 1% (David, personal communication).

The structural investigation of the F' variant was not limited to the characterisation of the substitution(s) responsible for the change in electrophoretic mobility. An important question to be asked relates to the cosmopolitan distribution of the known sequence and, in extension, to the true amount of variation that segregates at this locus. As discussed earlier, Kimura and Ohta (1975) and King and Ohta (1975) formulated models that presupposed electrophoretically distinguishable alleloenzymes containing a number of sequences incorporating silent and counterbalancing substitutions. Several investigators claim that this additional variation is indeed there, and can be revealed by the use of several techniques (Coyne, 1976; Singh et al, 1976). Is this the case for the ADH system? We feel that the only way this question can be answered is by sequencing, to the full extent, of the enzyme from /

from different sources. In addition, we attempted the survey of the sequence of ADH derived not from a single chromosome but from a population (in this case the Kaduna population from Nigeria).

The investigation of natural variants like ADH-F' and ADH-US is also an important approach to the determination of the structure and function relationships between the two major alleloenzymes. Such relationships can only be identified when the catalytic mechanism of the enzyme has been elucidated. Naturally occurring or artificially induced point mutations that affect the structural and kinetic behaviour of the enzyme can help identify residues important for catalysis or conformational stability and provide clues as to the secondary structure and mechanism of the enzyme.

No detailed investigation of the kinetic behaviour or of functionally important residues in *Drosophila* ADH has been conducted. Hence, in order to assist the identification of the effects of the substitutions in ADH-F', ADH-US or in other variants, we felt that at least a preliminary kinetic investigation was necessary.

A fundamental question is: on which properties is natural selection acting (if it is acting at all) to maintain the polymorphism in *Drosophila* ADH? Our variants appeared not to be under selection (Sampsell, 1977) but why was this so? Therefore information collected from the kinetic and structural studies has been /

been used, in conjunction with the existing knowledge on several dehydrogenases that catalyse similar reactions, in the formation of a theoretical outline of ADH function and the way the polymorphic nature of the enzyme has evolved.

CHAPTER 2: Materials and methods

2.1 Materials

(a) *Drosophila* food ingredients were obtained from the following sources:

Dried yeast: Distiller Co Ltd, UK.

Soybean flour: Soya Foods Ltd, Central House, Cambridge Road,
Barking, Essex, UK.

Molasses: Applefords Ltd, Poyle Close, Poyle Road, Colinbrook,
Slough, UK.

Malt extract: Potters Herbal Supplies Ltd, Douglas Works,
Leyland Mills Lane, Wigan, UK.

Cornmeal: Bonne Sante (Ldn) Ltd, 3 High Street, Chislehurst,
Kent, UK.

(b) For the purification of ADH the following materials were purchased:

Coomassie brilliant blue, G-250 and R-250: Sigma Ltd.

Nitro blue tetrazolium: Eastman Chemicals Ltd.

Phenazine methosulphate: Eastman Chemicals Ltd.

DEAE- /

DEAE-52, 23-cellulose:

Whatman Co Ltd.

Sodium dodecyl sulphate - especially

pure for electrophoresis:

Eastman Chemicals Ltd.

Agarose:

LKB Co Ltd.

(c) For sequencing work enzymes and chemicals used were obtained from the following suppliers:

Trypsin:

Worthington Biochemical Corporation,
Freehold, NY, USA.

Thermolysin:

Daiwa Kasei, KK, Osaka, Japan.

Pyridine (Nonin grade):

Rathburn Chem Ltd.

Ninhydrin:

Sigma Ltd.

Fluoroescamine:

gift from Dr J Hermoso.

Phenyl-isothiocyanate:

Rathburn Chem Ltd.

Trifluoroacetic acid:

Sigma Ltd.

(d) For kinetic work NAD^+ (89%, 99% pure), Thio- NAD^+ , 3-acetyl-pyridine-adenine-dinucleotide and pyrazole were purchased from Sigma Ltd.

Apart from the chemicals and various other materials mentioned above all others were purchased from BDH Chem Ltd.

2.2 The purification of Drosophila ADH

2.2.1 Strains and populations

The *Drosophila melanogaster* strains that were employed for the purification of ADH-F ("F prime") and ADH-US ("ultra slow") were kindly provided by their discoverer, J. David (University of Lyons, France), while the ADH-F and ADH-S flies were donated by B. Clarke (Dept. of Genetics, Nottingham University, U.K.).

The Kaduna population (from Nigeria) from which ADH-F was extracted (APPENDIX 1), was derived from the Kaduna population, maintained in the Department of Animal Genetics of Edinburgh University. At least 300 individuals were used to originate the population in the Department of Molecular Biology. The population was terminated after the end of the 2nd generation, when collection of adults and larvae had been completed.

The ADH produced by each strain was monitored by agarose gel electrophoresis at three monthly intervals, in order to avoid contamination with feral flies.

2.2.2 Growth medium

The flies were grown in a medium of the following composition:

80 g agar /

80 g agar
180 g dried yeast
100 g soybean flour
400 g molasses
800 g malt extract
800 g cornmeal

and about $8\frac{1}{2}$ l. of tap water bringing the total volume to 10 l.

The medium was prepared by first dissolving the agar in boiling water and immediately afterwards adding and mixing the rest of the ingredients.

The medium was autoclaved for 30' minutes at 17 lb/in² and after the removal from the autoclave 150 ml of acid mix was added, per 10 l. of medium, in order to reduce bacterial and fungal growth. The acid mix was prepared as follows:

836 ml of 100% proprionic were diluted with water to 1 l.

83 ml of 85% phosphoric acid were diluted with water to 1 l.

and two solutions were mixed.

2.2.3 Conditions of growth

The *Drosophila melanogaster* strains were kept in a room of constant temperature, air conditioned to $25^{\circ} + 1^{\circ}\text{C}$ with humidity kept constant at $50\% \pm 5\%$ by a rotating spray humidifier. A day/night switch controlled the lighting of the room. Under these conditions, the /

the generation time did not exceed 14 to 16 days, except for the ADH-US strain that was, on average, about two days slower when compared to the others.

2.2.4 Collection methods

Large quantities of flies are necessary for adequate yields of pure enzyme, for structural and kinetic experimentation. A starting batch of 250 g of larvae (or 100 g or adult flies) was regarded as standard. As time was a limiting factor all methods for the growth and collection of flies were conditioned by the need to maximise yields, while reducing the time necessary for the accumulation of the material.

Initially, milk bottles containing medium were each inoculated with five female flies and the appropriate number of males. The number of these "starting" bottles depended on the number of individuals present in the "stock" bottles. When the offspring had hatched they were transferred to a perspex population cage (100 cm x 60 cm x 60 cm). If not enough individuals were available - at least 300 were needed to initiate a "collection" size population - another generation was grown in milk bottles until a sufficient number was available. Food was inserted in the cages, in plastic trays (45 cm x 25 cm x 7 cm) and a dense population was allowed to build up.

At first, when adult flies were used for enzyme preparation,

a specially adapted vacuum cleaner was employed for harvesting. This method though was slow in producing the necessary amounts as a significant part of the population was removed before complete egg-laying had taken place. The next generation was therefore depleted in numbers and a period of up to a month was needed to build up the population again.

This difficulty was overcome by regular harvesting of the larvae of a population kept at constant density. When optimum density was reached food trays were inserted into the cage for egg laying. The trays - apart from those necessary for the continuation of the population - were removed after 24 hours, covered with muslin cloth and replaced with new ones. The eggs were allowed to develop to the 3rd instar larval stage.

Collection of the larvae proved to be more complicated than that of the adults. The food in the collection trays was suspended in tap water and passed through a wire sieve that retained the larvae, although some solid food particles were retained as well. The larval separation was achieved by floating the larvae in a 15-17% sucrose solution. In such a solution the solid food particles sink to the bottom. The floating larvae are again retained by the wire sieve, are washed with water and wrapped in aluminium foil. The batch is carefully weighed labelled and stored at -17°C . (Frozen larvae and adults gave adequate ADH yields after a period of four months). It was estimated that, providing the population density remained optimal, the necessary amounts for the preparation of /

of at least 100 mg of pure ADH can be collected in two months.

2.2.5 Purification procedure: conditions during, and steps

For structural studies on ADH, material was prepared by the method initially employed by Thatcher (1977), for the large scale purification of the enzyme. This method was subsequently modified (Thatcher, personal communication), mainly by the insertion of ammonium sulphate precipitation steps, before hydroxylapatite chromatography. The yields from the Thatcher method are poorer than for some of the other published procedures, but the ADH preparations are more stable.

Throughout the purification care was taken to keep the enzyme at cold room temperatures (4°C), and to complete the preparation as rapidly as possible.

The modified ADH purification comprises the following steps:

(a) Homogenisation

250 g* of larvae were blended with 100 ml of buffer (0.1 M Tris-HCl, /

* Quantities represent a typical ADH purification with a starting material of 250 g of larvae.

(0.1 M Tris-HCl, pH 8.7, containing 1 mM EDTA and 0.1 M benzamidine as protease inhibitors (Mares-Guia, 1968)) in a Waring blender. The blending was done in 1 minute bursts at maximum speed, while in the intervals the blender container was cooled in ice. The homogenisation was completed when adequate cell disruption had occurred, defined by the absence of intact cells in samples of homogenate examined under a microscope. Insoluble cell debris was removed by centrifugation of the homogenate at 23,000 g at 4°C for 30 minutes in a MSE High Speed 18 centrifuge. The supernatant was subsequently passed through a double layer of muslin to filter off a cake of floating fatty aggregates.

(b) Protamine sulphate treatment

The crude extract was transferred to a beaker and was kept cool in ice. Nucleic acids and related compounds were removed by protamine sulphate or streptomycin sulphate precipitation. These were added slowly, under stirring, as 10% aqueous solutions - approximately 3 g of precipitant were used for 100 g of starting material. The mixture was continuously stirred for 10 minutes. The precipitate was removed by centrifugation (at 23,000 g at 4°C for 30 minutes). In this step, because of the reduced viscosity of the solution, the cloudy suspension of mitochondria that had not been sedimented in the previous step was finally removed.

(c) Ammonium sulphate fractionisation

At first the solution was brought to 40% ammonium sulphate saturation /

saturation by adding the calculated weight of the compound in solid form. It was added gradually, with slow stirring, in order to avoid high local concentrations of the salt. The pH of the solution was not controlled, but did not drop below pH 5.5. The small amount of precipitate was removed by centrifugation (23,000 g at 4°C for 30 minutes) and was discarded.

The ammonium sulphate saturation of the supernatant was then raised to 60% by further addition of the solid. The precipitate was collected after centrifugation and the supernatant was discarded.

(d) Desalting

The 40-60% saturation precipitate was resuspended in 0.05 M Tris-HCl buffer of pH 8.7 and the salt was removed either by dialysis against the buffer or by gel filtration (in which case the volume after resuspension was kept below 100 ml), through a Sephadex G-25 coarse column (diameter 7.5 cm, height 30 cm). The gel filtration option was usually chosen, as dialysis was time consuming (up to 48 hours) and had adverse consequences on the final yield.

(e) Hydroxylapatite chromatography

This was a step designed to remove the protease that co-purifies with ADH in most separation systems, and which significantly affects the half-life of the enzyme in purified preparations. The presence of this protease was obviously responsible for the underestimation of the molecular weight of the sub-unit by Jacobson and Pfuderer (1970), and was also recognised by Schwartz et al (1975) and Thatcher (1977). /



by Schwartz et al (1975) and Thatcher (1977).

The column (diameter 5 cm, length 5 cm) was equilibrated with 0.05 M Tris-HCl, pH 8.7 and great care was taken to ensure that both sample and eluant conductivity did not exceed $35 \Omega^{-1} \text{m}$. After passing the sample through, the column was washed with a bed volume of buffer and the effluent was collected.

(f) Gel filtration

The protein of the sample at the end of step (e) was concentrated by ammonium sulphate precipitation at 60% saturation. The precipitate was collected by centrifugation under the known conditions, and the pellet was redissolved in as small a volume as possible (kept below 20 ml) of 0.1 M Tris-HCl pH 8.7 buffer (containing 1 mM EDTA and 0.1 mM benzamidine) as sample size crucially affects purification in this step.

The sample was then applied on a Sephadex G-100 fine column (diameter 5 cm, length 140 cm), equilibrated in the above mentioned buffer. The flow rate was kept constant at about 40 ml an hour with a peristaltic pump. 7.5 ml fractions were collected, with the ADH activity peak appearing after 1,400 ml (in about 35-48 hours). The void volume was about 350 ml.

(g) Freeze-drying

The fractions that contained the central area of the ADH activity peak were pooled and purity was assessed (section 2.2.9). Although /

Although most of the protease is removed during the hydroxylapatite chromatography step, contamination can only be eliminated during the gel filtration. If the purity criteria show that the protease level is still high enough to affect structural investigation, then gel filtration must be repeated.

When purity criteria are satisfied, the protein is again concentrated by ammonium sulphate precipitation at 60% saturation of the solution, and centrifugation. The pellet is then dissolved in 5-7 ml and desalted by gel filtration through a column (diameter 2.5 cm, length 30 cm) of Sephadex G-25 fine, equilibrated with a volatile buffer (0.1 M ammonium carbonate). The conductivity of the salt-free solution of the enzyme resulting from the filtration is brought to below $30 \Omega^{-1} \text{m}$ and the protein is freeze-dried. The solid is kept under vacuum at -17°C , but even under these conditions it slowly deteriorates.

2.2.6 Modification for large batches

In several cases the amount of starting material was larger than 250 g (in larvae). In these preparations certain steps were adapted in order to deal with the increased quantities. For desalting the ADH fraction after ammonium sulphate fractionisation (step d), dialysis was either preferred or it was coupled to gel filtration. For the hydroxylapatite chromatography, where retention of the contaminant protease is weak, 1.5 cm in height was added to the height of the column for each additional 100 g of larvae.

Duplication /

Duplication of the Sephadex G-100 F filtration step was necessary as the maximum sample volume was invariably exceeded in the first attempt.

2.2.7 Additional purification steps

Hydroxylapatite chromatography (step e) is essential in the purification strategy because of its effectiveness in removing the contaminant protease. However, it cannot always be used, as some of the more basic ADH variants (ADH-S, ADH-US) are absorbed in it. Retention of these variants is weak (as with the protease) and even shallow salt or buffer concentration gradients fail to give adequate separation. For ADH-S and ADH-US successful removal of the protease can only be accomplished by the repeated careful use of the Sephadex G-100 F filtration step.

Variation in absorption properties of different batches of hydroxylapatite (even from the same manufacturer) also affected the reproducibility of purification.

DEAE-52 or DEAE-23 cellulose retain both protease and ADH though not the S or US variants in buffers with low conductivities ($30-35 \Omega^{-1} \text{m}$). Retention is weak and protein concentration and bed volume influence the results. A shallow salt (NaCl) concentration gradient gives a separation, albeit not a perfect one. DEAE cellulose chromatography was therefore inserted occasionally to the preparation procedure described above. However, it was usually employed /

employed in the purification of ADH-S and US for the reduction of soluble protein content before step F (Sephadex G-100 F gel filtration).

DEAE-52 was preswollen and could be used immediately after the removal of the fine particles. DEAE-23 had to be precycled by a number of successive alternating washes in 1 M NaOH and 1 M HCl before equilibration with the desired buffer.

2.2.8 ADH activity assay

ADH activity was monitored during purification by following the NAD^+ reduction spectrophotometrically at 340 nm using a Pye-Unicam SP 1800 double beam recording spectrophotometer at room temperature (20°C).

The standard assay was performed in a quartz 1 cm semi-micro cuvetted (capacity 1 ml). To 1 ml of 100 mM isopropanol in the reaction buffer (0.1 M Tris-HCl pH 8.7), 10 μl of 0.1 M NAD^+ was added bringing the total NAD^+ concentration to 10^{-3} M. The reaction was started by the addition of 10 μl of the enzyme solution. The rate of NADH formation was recorded and the initial velocity was estimated from the tangent of the progress curve at its origin.

2.2.9 Protein concentration assays

Two methods were usually employed for the estimation of protein /

protein concentration in the purification fractions.

(a) The Lowry method as modified by Miller (1959), calibrated with Bovine Serum Albumin.

(b) The Bradford method as modified by Bearden (1978). Calibration was again done with Bovine Serum Albumin. In this assay, Coomassie brilliant blue G-250 is used as the reactant. (Reaction solution: 1 mg Coomassie brilliant blue/ml 85% (w/v) phosphoric acid diluted five fold-200 ml/l - with water). The protein solution to be assayed was mixed with an equal volume of the reaction solution. Protein concentration was determined by the increase in absorption at 595 nm in plastic cuvettes (the protein-dye complex binds to quartz).

The relative response of the Lowry assay for ADH is very similar to that displayed for BSA. No estimates are available for the Bradford method which was generally preferred due to speed of the procedure.

2.2.10 Criteria of purity of ADH preparations

Purity of ADH preparations was monitored by gel electrophoresis under denaturing and non-denaturing conditions.

(a) Sodium-dodecyl-sulphate (SDS) polyacrylamide gel electrophoresis

This was performed in slab gels in a vertical electrophoresis apparatus /

apparatus (Raven Scientific). The discontinuous buffer system devised by King and Laemmli (1971) - Table 2.1 - was used. A polyacrylamide concentration gradient, ranging from 9% (w/v) at the top, to 27% (w/v) at the bottom was also used to provide improved resolution of the protein bands in the gels. Electrophoresis was conducted overnight under standard current of 9 mA. Progress was followed by the movement of the dye (Bromophenol blue) that was included in the sample buffer ingredients. When the dye reached the bottom of the gel electrophoresis was complete. The gel was then removed and placed in a methanol/acetic acid/water (5:1:5 v/v) solution that fixed the protein bands and removed the SDS. (Removal of the SDS was thought necessary because it interfered with the effectiveness of the staining solution). The gels were stained in the same solution now containing 0.1% (w/v) Coomassie brilliant blue R-250. Staining was allowed to proceed for up to 4 hours. The gels were destained in a 10% acetic acid (v/v) aqueous solution.

(b) Agarose gel electrophoresis

This was performed on thin layer (2-3 mm) agarose slab gels (agarose content 1%). The buffer employed in the gels and troughs was 0.1 M borate pH 8.9 (Ursprung and Carlin, 1968). Samples were loaded in the middle of the gel, along the longitudinal axis. Electrophoresis was performed on the LKB 2117 Multiphor flat gel electrophoresis apparatus. The gel support plate was cooled by water circulated at 4°C. The progress of the electrophoresis was monitored by the movement of a dye solution (0.5% w/v Bromophenol blue /

Table 2.1 : Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis - buffers and solutions.

Lower gel buffer

22.7 g Tris
10 ml 5% SDS aqueous solution
Volume is brought to 100 ml with H₂O
pH is adjusted at 8.8 with HCl

Upper gel buffer

6 g Tris
8 ml 5% SDS aqueous solution
Volume is brought to 100 ml with H₂O
pH is adjusted at 6.8 with HCl

Tank buffer (concentrated)

12 g Tris
57.8g Glycine
4 g SDS
Volume is brought to 1 lt with H₂O
Before use, the concentrated tank buffer is diluted by the addition of 3 volumes of H₂O to 1 volume of the prepared solution

Sample buffer

0.75 g Tris
2 g SDS
10 g sucrose
5 ml 2-mercaptoethanol
Volume was brought to 100 ml with H₂O
pH is adjusted at 7.0 with HCl

Polyacrylamide concentration gradient (9-27%) gel

	<u>Lower gel solutions</u>		<u>Upper gel</u>
	9%	27%	
Lower gel buffer	3 ml	3 ml	-
Upper gel buffer	-	-	5 ml
40% acrylamide aqueous solution (w/v)	3.75 ml	12	1.25 ml
3% bis-acrylamide aqueous solution (w/v)	1.5 ml	1.5 ml	4 ml
TEMED	10 μ l	10 μ l	25 μ l
Ammonium persulphate solution (10 mg/ml)	250 μ l	250 μ l	500 μ l
Water	8.25 ml	-	9.7 ml

Non- gradient gel (polyacrylamide concentration=16%)

	<u>Lower gel solution</u>	<u>Upper gel</u>
Lower gel buffer	10.0 ml	
40% acrylamide aqueous solution (w/v)	16.0 ml	
3% bis-acrylamide aqueous solution (w/v)	1.23 ml	as above
TEMED	20 μ l	
Ammonium persulphate solution (10mg/ml)	700 μ l	
Water	12.66 ml	

blue in water). Current was kept at 20 mA as higher current caused the gel to overheat.

Afterwards the gel was carefully removed from the supporting plate and stained. Staining, for the identification of protein bands, was achieved in the way described for the SDS gels. ADH bands were identified by activity staining of identical samples run in parallel. For ADH activity staining the following procedure was adopted.

The gel was transferred to a container and soaked in reaction solution that consisted of:

100 ml of the reaction buffer (0.1 M Tris-HCl pH 8.7)

25 mg of NAD^+

25 mg of Nitro blue tetrazolium

5 mg of Phenazine methosulphate

The staining container was then sealed and incubated at 37°C for 30 minutes. ADH activity bands appear as blue spots with ADH-5 developing almost immediately. ADH-3 and ADH-1 development is slower. The gels were then fixed in the methanol/acetic acid/water solution.

Another purity criterion, less sensitive than the electrophoretic methods described above was provided by the amino acid analysis of ADH preparations. The amino acid composition of the pure ADH is known (Thatcher, 1977, 1980; Benyajati et al, 1981) but such amino acid analysis is not a very accurate tool for the estimation /

estimation of the degree of purity of the samples.

2.2.11 Protease activity assay

The position of the contaminant protease in the salt concentration gradient elution profile after DEAE cellulose chromatography was determined by a dye release assay.

Aliquots of 50 μ l from each fraction were added to a series of 1.5 ml polyethelene microcentrifuge tubes containing 1 ml of azoalbumin solution (20 mg/ml) in Tris-HCl pH 8.7 buffer. The tubes were then sealed and incubated at 37°C overnight. After 12-18 hours the protein was precipitated by the addition of an equal volume of 5% Tri-chloroacetic acid (TCA) aqueous solution. The precipitate was removed by centrifugation at 20,000 g. Protease activity was assessed by the amount of free dye in the supernatant measured against the blank at 580 nm.

A very sensitive visualisation of the contamination of the preparations by this unidentified protease was provided by the appearance of low molecular weight bands in the SDS polyacrylamide gels when samples were incubated at non-denaturing temperatures before additions in the gel wells.

2.3 /

2.3 Sequence methods

2.3.1 Performic acid oxidation

Performic acid oxidation (Hirs, 1956) was employed for the denaturation of the enzyme before enzymic digestion.

The performic acid used was prepared by adding 0.5 ml of 30% aqueous hydrogen peroxide solution in 9.5 ml of anhydrous formic acid. The mixture was stirred with a glass rod and incubated at room temperature for at least one hour.

The freeze-dried protein is dissolved in this preformed performic acid (1 ml performic acid/10 mg protein). The reaction vessel is then placed in ice and the reaction is allowed to proceed for 1 hour. Since methionine is not present in *Drosophila* ADH composition, even shorter reaction times are adequate (Hirs, 1956).

At the end of the reaction water is added bringing the acid concentration to 5% (v/v) and the solution is freeze-dried.

2.3.2 Enzymic digestion

The oxidised protein was dissolved in 8 M urea and then three volumes of 0.2 M (in respect to NH_4OH) ammonium acetate buffer pH 8.7 are added to result in a solution of 2M urea, at which concentration trypsin and thermolysin remain active.

Trypsin, /

Trypsin, treated with diphenyl carbonyl chloride (Erlanger and Cohen, 1963), or thermolysin were added to the solution at a ratio of 1/40 (w/w) enzyme to substrate. Digestion was allowed to proceed for 5-18 hours at 37°C.

After digestion the sample was diluted with water to bring the conductivity below 35 mΩ⁻¹.m (high salt concentration interferes adversely with lyophilisation) and the solution was again freeze-dried.

2.3.3 Peptide fractionisation

The freeze-dried digest was dissolved in 1 ml of 1 M acetic acid in an effort to keep sample volume for the subsequent gel filtration to a minimum.

The sample was loaded on a Sephadex G-25 SF column (length 50 cm, diameter 1.5 cm), equilibrated with 1 M acetic acid and operating under gravity. The effluent was collected in fractions of 1.5 ml in volume. Protein content in the fractions was monitored at 254 nm by an LKB Uvicord recorder.

The number and position of the peptides along the elution profile was then determined by High Voltage Paper Electrophoresis (HVPE). On a 3 MM Whatman paper, 100 μl from each fraction were applied, in successive 10 μl applications, at spatial intervals of 1 cm. The paper was then subjected to electrophoresis in a volatile buffer of pH 6.5. (Further details on paper electrophoresis in section /

section 2.3.4). When the electrophoresis was complete the paper was dried and examined under UV light (340 nm wavelength) for tryptophan containing peptides (oxidised tryptophan peptides fluoresce strongly). These are distinctively marked with a pencil and the paper is subsequently stained with ninhydrin or fluorescamine (section 2.3.4). The stained peptides were again marked, and immediately afterwards histidine and tyrosine containing peptides were identified by Pauly staining (section 2.3.4). After the final development of the electrophoretogram, fractions were collected into separate pools (the pools being chosen so as to simplify the purification of the peptides from the digest) and were freeze-dried.

2.3.4 Preparation paper electrophoresis and chromatography

The lyophilised fraction pools were dissolved in a small amount of 1 M acetic acid and the peptides were purified by the following techniques.

(a) High Voltage Paper Electrophoresis (HVPE)

It was performed in glass solvent cooled tanks (Michl, 1951). White spirit ("varsol") was used as the coolant, except for the pH 6.5 tank for which toluene was employed. Cooling during electrophoresis was achieved by tap water running through stainless steel piping attached below the cover of the tank and immersed in the coolant.

The /

The following buffers were employed:

	Pyridine	:	Glacial acetic acid	:	water
pH 6.5	100	:	4	:	900

(Ryle, Sanger, Smith and Kitai, 1955).

	Pyridine	:	Glacial acetic acid	:	water
pH 3.5	1	:	10	:	89

(Ryle, Sanger, Smith and Kitai, 1955).

	Glacial acetic acid	:	Formic acid	:	water
pH 2.1	4	:	1	:	45

(Ambler, 1963).

Whatman 3 MM paper was used, of the standard length of 57 cm. The width of the paper varied with the number of samples, but could not exceed 46 cm.

Samples were dissolved in volatile buffers and were applied at loadings not exceeding 5 μ M/cm. Amino acid and coloured markers were always used. These were "wondermix" (Milstein, 1966), methyl green and the "red pentel pen" dye. Peptide mobilities were measured against these markers (Offord, 1966). The voltage employed (constant throughout the experiment) was 3 kV, giving a potential gradient of 65 V/cm. The progress of the electrophoresis was followed by the movement of the methyl green (for pH 3.5 and 2.1) and red ink (for pH 6.5), placed at the edges of the loading line and clearly visible through the glass tank.

In /

In the preparative version of this technique, small amounts of the sample are spotted on the flanks of the major sample spot. After electrophoresis, the strips of paper containing these flanking samples are cut off and stained. By aligning these with the rest of the paper the constituents of the major sample can be identified without directly staining it.

(b) Paper chromatography

Descending paper chromatography was performed in glass tanks (22 cm x 50 cm x 57 cm). The developing solvent was contained in a glass trough, fixed at the top of the tank, and had the following composition:

Butan-1-ol : Glacial acetic acid : water : Pyridine
 15 : 3 : 12 : 10 (v/v)
 (Waley and Watson, 1953).

The samples were spotted on a Whatman 3 MM paper in the same way as for paper electrophoresis. Descending chromatography was allowed to proceed for at least 18 hours (progress was followed by the movement of the methyl green spot). Since impurities present in the same grades of commercially available pyridine interfere with ninhydrin staining a special grade of this chemical was employed (Rathburn Chemicals). No special care was taken to remove aldehydes from the n-butanol preparations.

(c) Peptide and amino acid staining methods /

(c) Peptide and amino acid staining methods

(1) Ninhydrin staining

It was regularly employed for the visualisation of peptides and amino acids contained in paper electrophoretograms. The standard staining solution was composed of 0.2% ninhydrin in acetone in which a few drops of 50% 2,4,6 - collidine in acetic acid, were placed. Staining was achieved by dipping the paper in a trough containing the staining solution. The paper was then allowed to dry at room temperature. For the full development of the stain, 1-2 minutes incubation at 105°C was necessary, although some peptides with n-terminal valine or isoleucine require longer development time at room temperature.

(2) Pauly staining (Dent, 1947)

It was employed for the identification of histidine and tyrosine containing peptides. It usually followed ninhydrin staining, so ninhydrin positive areas identified by the previous technique were clearly marked before it was performed.

A solution of equal volumes of the following reagents:

1% Sulphanilic acid in 1 M HCl

5% NaNO_2

was incubated at room temperature for 10 minutes and was afterwards sprayed onto both sides of the paper until all traces of the ninhydrin staining had disappeared.

The /

The paper was then allowed to dry and was subsequently sprayed with 15% Na_2CO_3 . Histidine containing peptides gave a strong red colour. Tyrosine peptides appeared as brown spots.

(3) Fluorescamine staining (Mendez and Lai, 1975)

It was used only when small amounts of peptides were present or for peptides that gave a weak ninhydrin reaction. It was also used to allow accurate cutting out of close run peptide bands after preparative HVPE.

Staining was achieved by dipping the paper in a solution of 1% N,N-ethylmorpholine in order to buffer the paper at pH 10. The paper was then allowed to dry and was subsequently redipped in a fluoroescamine solution (10 mg/ml in acetone).

For the identification of peptides in preparative HVPE, the reagents were sprayed onto the paper with an atomiser. (Both fluoroescamine and ninhydrin achieve staining by reacting with the free amino groups of the peptides. Peptides thus treated cannot be used for sequencing work. When sprayed a weaker fluoroescamine solution is employed (1 mg/ml acetone) so as to only react with a low proportion of the peptide).

After drying, the paper is examined under UV light (long and short wavelength, 340 and 254 nm) where peptides fluoresce strongly.

2.3.5 Elution of peptides /

2.3.5 Elution of peptides

After preparative paper electrophoresis, the peptides were eluted from the supporting paper phase with 0.1 M NH_4OH in elution towers. Standard apparatus described by G. Allen (1981) and elsewhere. About 0.2 ml/cm^2 of eluant were allowed to run through the paper strips and elution was usually completed in $2\frac{1}{2}$ -3 hours.

The eluted peptides were subsequently dried in vacuum over sulphuric acid, redissolved in a known volume of 1 M acetic acid and stored at -17°C .

2.3.6 Amino acid analysis

The amino acid composition of the purified peptides of the digests, or of the intact protein was determined as follows: the first step consisted of qualitative amino acid analysis, designed to give a rough idea about the residues involved, as well as help identify cysteine containing peptides (transformed to cysteic acid in the oxidised protein). This is essential, since in the qualitative amino acid analysis technique that was used cysteic acid was not recognised.

For qualitative amino acid analysis, 5-10 nM of peptide were hydrolyzed in $50 \mu\text{l}$ 6N HCl in a heat-sealed Durham tube (diameter 8 mm, length 35 mm) for 18-24 hours at 105°C . The tubes were then opened and the acid was removed in vacuum over NaOH.

The /

The amino acids were identified by HVPE in pH 2.1 using mixtures R and T (Table 2.2). The collidine-acetone-ninhydrin staining helped considerably in amino acid identification because of the differences in colour given by different amino acids.

Samples were then prepared for quantitative amino acid analysis. An amount of peptide solution containing 25-50 nM of peptide was dispensed into a tube (diameter 10 mm; length 120 mm) and dried. Then 0.2 ml of water and 0.2 ml of HCl (Aristar grade, BDH Chemicals) were added. The tubes were drawn out with an oxygen gas flame, evacuated (6.1 mm torr) and heat-sealed. Hydrolysis was allowed to proceed for 24 to 36 hours at 105°C. Longer hydrolysis times were employed for the correct estimation of isoleucine and valine residue content of peptides and protein.

For the determination of amino acid composition of the thus prepared samples from peptides resulting from the enzymic digestion of the oxidised ADH molecule, a Rank Hilger Chromaspek amino acid analyser was used. A Digico 16 V computer and a teletype were interfaced to the analyser and provided printouts showing amino acid recognition of elution peaks, retention times, peak area, and amino acid concentration in nM/ml sample. But the routine was occasionally upset by variations in retention times beyond the "window" allowed (caused by fluctuations of column pressure and the ageing of the ion-exchange resin) resulting in false recognition, and therefore in erroneous integration of the peaks. So in a variety of cases, integration had to be done by the operator, using the computed /

Table 2.2 : Markers employed in High Voltage Paper electrophoresis

General purpose marker "Wondermix" (*)

Lysine
Histidine
Arginine
Glycine
Valine
Alanyl glycine
ε-dinitrophenyl lysine (yellow)
taurine
cysteic acid
aspartic acid
glutamic acid

Amino acid markers employed in qualitative amino acid analysis (†)

R	T
Lysine	Histidine .
Arginine	Glycine
Valine	Alanine
Leucine	Serine
Proline	Isoleucine
Phenylalanine	Threonine
Tyrosine	Glutamic acid
Methionine Sulphone	Aspartic acid
Cysteic acid	ε-dinitrophenyl lysine (yellow)

(*) The order of presentation is the order of separation from cathode(-) to anode (+) on electrophoresis at pH 6.5

(†) The order of presentation is the order of separation from cathode(-) to anode (+) on electrophoresis at pH 2.1

computed peak area and amino acid concentration/unit area ratios.

Errors in computer integration of overlapping peaks were unavoidable because of the routine employed by the computer (supplied by the manufacturer) for the estimation of peak areas. Errors were significant when one of the overlapping peaks was much larger than the other. Arginine integration (eluted closely after ammonia) was thus particularly affected.

Cysteic acid was not measured using our procedure on the Chromaspek analyser as this amino acid is not retained at all by the resin, and so is lost in baseline changes at the start of the analysis as a broad peak. Because of these in-built limitations the Chromaspek analyser cannot be used successfully for the correct estimation of amino acid composition of large peptides and proteins. In such cases, a Beckman 120 C amino acid analyser with a two column system was used. Integration of peaks was done manually by the operator.

Both analysers used Nor-Leucine as the internal standard and ninhydrin as the colour developing agent. Elution was monitored at 570 nM for the amino acids, and at 440 nM for proline.

2.3.7 N-Terminus identification

For the identification of the N-terminal amino acid of the peptides the Gray and Hartley method (1963) of sample preparation was used.

The /

The sample (10-20 nM) was dispensed into a Durham tube and dried over sulphuric acid. Then 10 μ l of 0.1 M NaCO_3 were added, to displace ammonia and provide subsequent buffering, and the tubes were dried again. Afterwards 10 μ l of dansyl chloride solution (2.5 mg/ml acetone) and 10 μ l of water were added and mixed. The tubes were covered with parafilm (to keep the acetone in) and incubated at 37°C until the yellow colour of the solution disappeared (approximately 45 min). The contents were again dried in vacuo.

Hydrochloric acid was then added (50 μ l, 6N) and the tubes were heat-sealed and incubated at 105°C for approximately 18 hours. After hydrolysis the tubes were opened and the contents were dried in vacuo over NaOH.

The dansyl-amino acid resulting from this treatment was identified by multi-dimensional thin layer chromatography on small polyamide sheets (Wang, 1967). The dried sample was dissolved in 2-4 μ l of absolute alcohol (ethanol) and applied as a small spot (diameter 1 mm) onto both sides of a 5 cm x 5 cm square polyamide sheet (Cheng-Shin Trading Co & BDH Chemicals). On one side only 1 μ l of ethanol solution of several Dns-amino acids was applied on to the sample spot (the standard solution contains bis-Dns-lysine, Dns-isoleusine, Dns-proline, Dns-alanine, Dns-glutamic acid, Dns-threonine and Dns-arginine).

The Dns-amino acids were separated in the solvent system listed below:

1st dimension: /

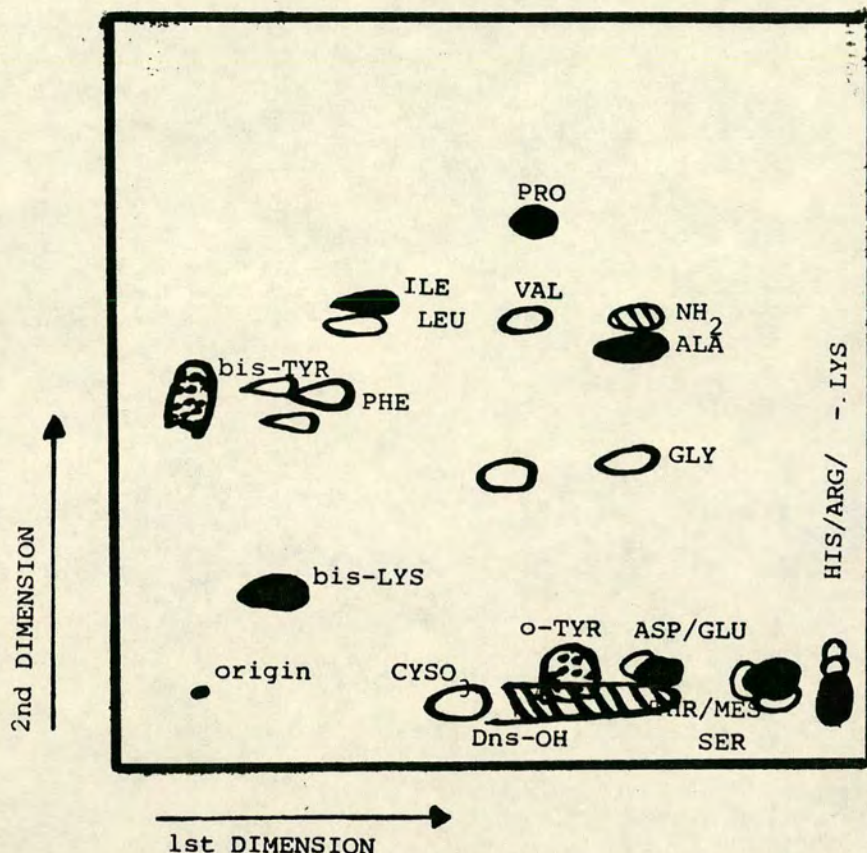





Fig. 2.1.a: Dns-amino acid separation after the second dimension of polyamide sheet thin layer chromatography

 = BLUE

 = YELLOW

 Dns-amino acids of the standard solution

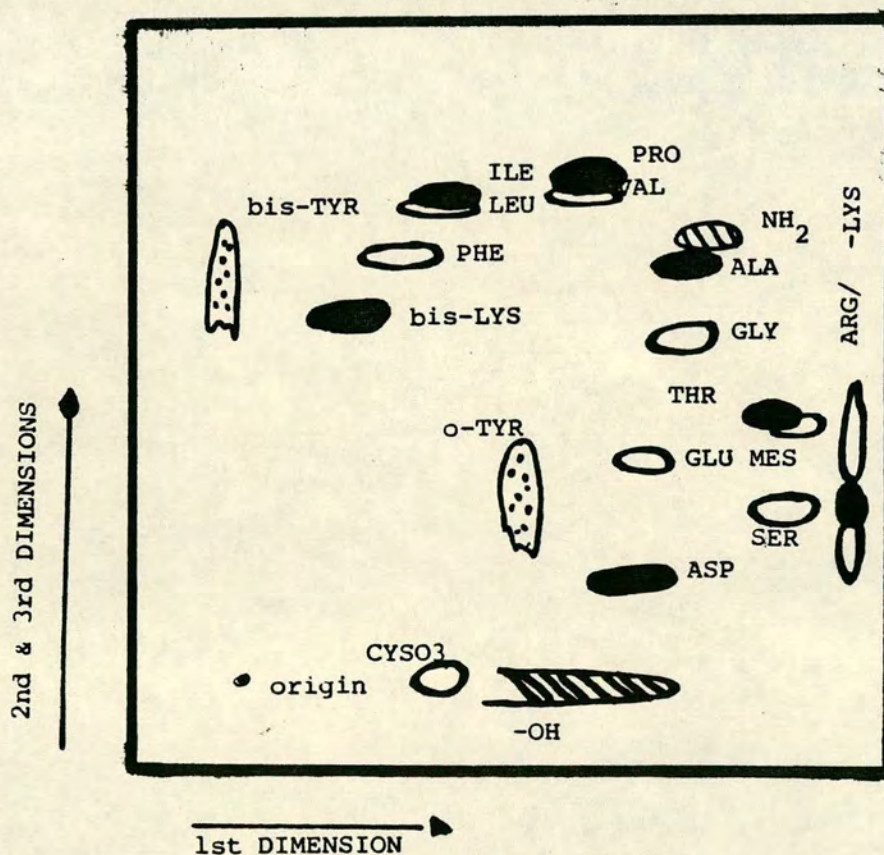


Fig. 2.1.b: Dns- amino acid separation after the second and third dimension of polyamide sheet thin layer chromatography

1st dimension: 5% formic acid (Scheffer, 1973)

2nd dimension: toluene : acetic acid (9:1 v/v), run at right angles to the first dimension

3rd dimension: butyl acetate : methanol : acetic acid (3:2:1 v/v) run in the same direction as the second dimension (Woods and Wang, 1967).

On all occasions the plate was removed from the chromatography tank when the solvent covered 7/8 of the surface of the polyamide sheet.

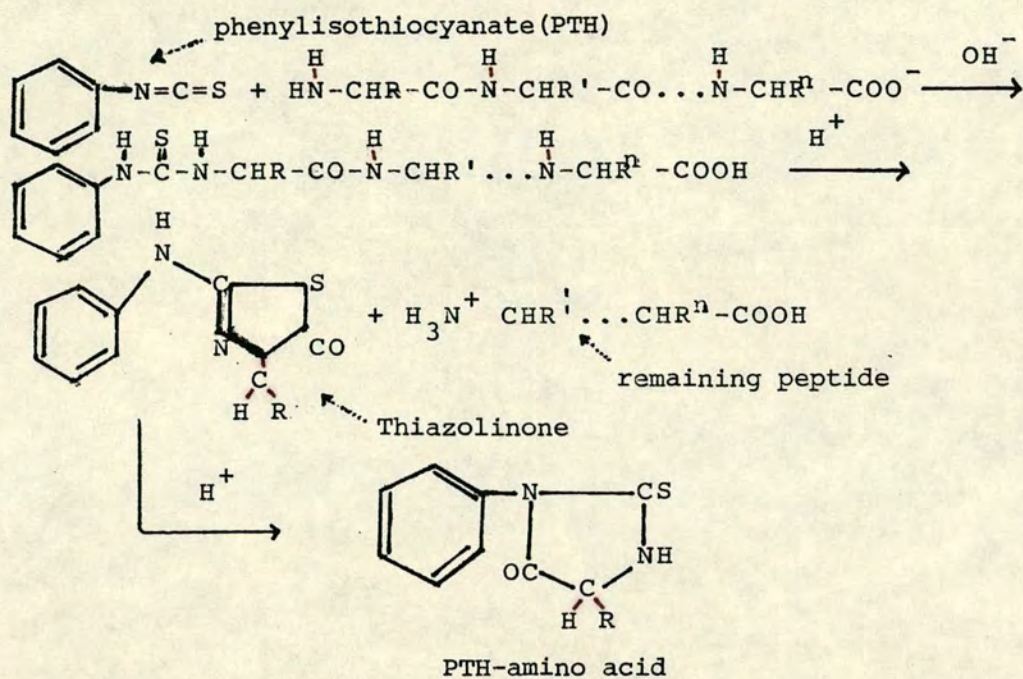
After all dimensions were run the Dns-amino acid of the sample was identified by its position relative to one of the Dns-amino acids of the standard solution. (Fig. 2.1.a and 2.1.b show Dns-amino acid separation after the 2nd and 3rd dimensions respectively).

Polyamide sheets were cleaned in a 50% acetone solution in 0.1 M acetic acid and could be used successfully for about 10 times. As they aged, mechanical damage and chemical deterioration resulted in uneven movement of the solvent front during chromatography.

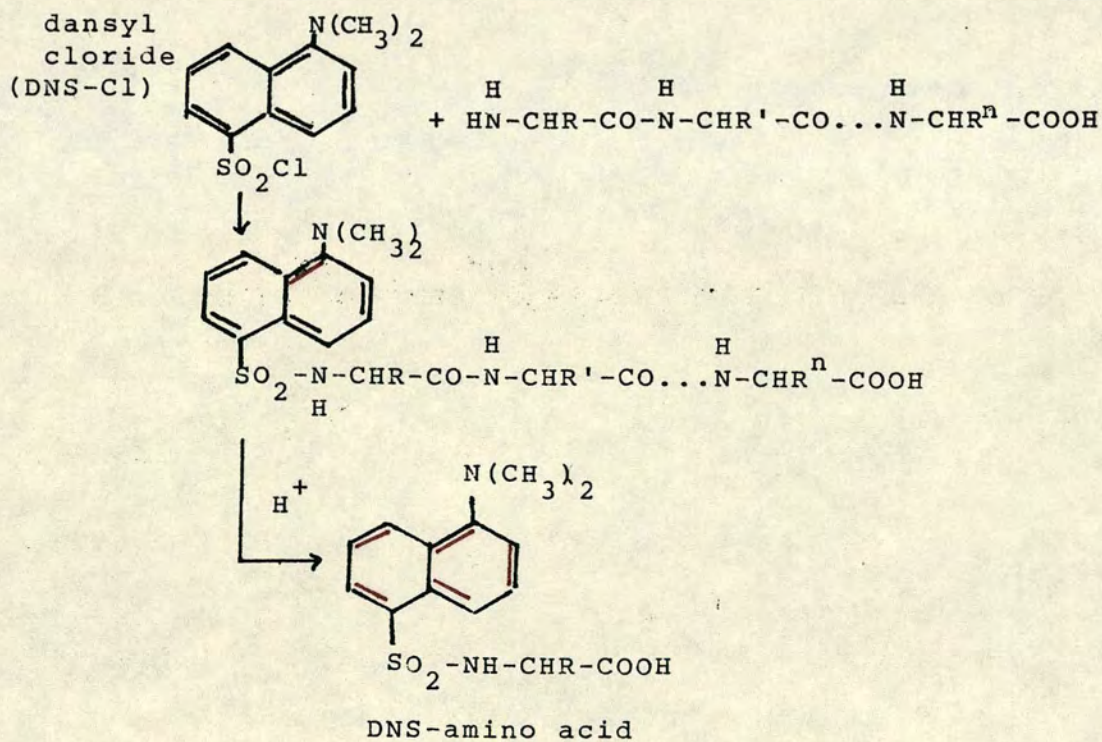
2.3.8 Edman sequential degradation of peptides

An aliquot of the peptide solution (optimal content is around 10 nM of peptide for each proposed cycle) was dried down in a custom made screw cap tube (diameter 12 mm; length 65 mm).

Aqueous /



Reaction scheme 1 : Edman degradation of peptides.



Reaction scheme 2 : The dansyl chloride method.

Fig. 2.2 : Reaction schemes for the Edman sequential degradation and dansyl chloride methods.

Aqueous solution of pyridine (0.2 ml of 50% v/v) and 0.1 ml of 5% (v/v) phenyl-isothiocyanate in pyridine were added.

The tubes were gassed with oxygen-free nitrogen, capped and incubated at 37°C for 1 hour. The contents then dried in vacuo in a hot desiccator (temperature kept at 60°C). When cooled 0.15 ml of trifluoroacetic acid were added and the contents were again gassed with oxygen-free nitrogen and incubated at 37°C for 30 minutes. The trifluoroacetic acid was then removed in vacuo, over sodium hydroxide.

Water (0.25 ml) was added and three butyl acetate extractions were performed. (2 ml of butyl acetate were added in each tube and the contents were vortex mixed. The solution was then centrifuged and the upper non-aqueous phase was removed with a syringe and discarded). The aqueous solution was then dried overnight.

When dry it was redissolved in 50% pyridine (0.2 ml) and a sample was removed. The volume of the sample varied as to which cycle of the degradation procedure we arrived at and transferred into a Durham tube. A corresponding amount of pyridine solution was added, bringing the volume again to 0.2 ml and a new cycle began.

The phenyl-isothiocyanate and trifluoroacetic acid treatment removed the N-terminal amino acid of the peptide (reaction schemes shown in Fig. 2.2). The newly unmasked N-terminus was identified with the Dansyl chloride method described in section 2.3.7.

2.4 Estimation of several kinetic constants and kinetics
 of thermal denaturation

2.4.1 Isolation of ADH-5 isoenzyme of the S,F,F' alleloenzymes

Since there are appreciable differences among the kinetic and physical properties of the ADH isoenzymes (Day and Needham, 1974) only the ADH-5 form of the alleloenzymes used (F,S,F') was employed for the determination of kinetic constants.

ADH-5 was isolated from the usual mixture of all three forms, resulting from the previously mentioned purification method (section 2.2), by affinity chromatography. For that purpose an 8-hexyl-6-amino-AMP Sepharose-4B column (described by Leigh-Brown and Lee, 1979) was employed (diameter 2.5 cm; length 30 cm).

The column was equilibrated with 0.05 M phosphate buffer pH 6.0 and the sample was added. The column was then washed with two bed volumes of buffer. ADH-5 was eluted by passing through the column a 1 bed volume of 10^{-4} M pyrazole in a 5×10^{-4} M NAD^+ solution in buffer.

The separation was confirmed by agarose gel electrophoresis. Affinity chromatography was also the most effective way of removing all traces of the contaminating protease. But retention is weak (the 2.5 cm; 30 cm column can successfully retain about 7 mg of ADH-5) and large scale purification of the enzyme by this method is impractical.

Purified /

Purified ADH-5 was kept in solution, since it had been discovered that freeze-drying partially denatures the enzyme (sections 3.1 and 4.1). The solutions (in the above mentioned buffer are surprisingly stable (retain almost 100% of activity for a week if kept at 4°C). Freezing and thawing of such solutions also denatures the enzyme and was therefore avoided.

2.4.2 Kinetics of ADH

(a) Conditions and equipment

Spectrophotometric measurements were performed with a Perkin-Elmer 320 spectrophotometer. On this instrument absorbance scale, chart speed and response time could be varied by the operator in almost any combination so as to be able to obtain almost linear recordings of the reaction rate at the beginning of the progress curve. This flexibility and sensitivity of the instrument greatly assisted in the more accurate calculation of the initial velocities.

The values of initial velocities employed for the estimation of the Michaelis-Menten constants and maximal velocities were the averages of at least two, but in the overwhelming majority of cases, of three measurements.

Measurements for the examination of pH dependencies of kinetic constants of the forward reaction were performed following the procedure listed below.

Substrate /

Substrate solution (0.98 ml) in the appropriate buffer was pipetted into a 1 ml (optical path 1 cm) semi-micro quartz cuvette.

NAD⁺ solution (10 μ l) was added (10^{-1} M in buffer) bringing the total NAD⁺ concentration to 10^{-3} M.

The cuvette contents were then stirred with a plastic rod and their absorption at 340 nm was brought to zero by the use of the "zeroing" function of the instrument.

Enzyme solution (10 μ l) was then added, the cuvette contents were again stirred and the reaction progress was monitored at a wavelength of 340 nm.

Assays for NAD⁺ and NAD⁺-analogs kinetics were performed as follows:

The desired concentration of NAD⁺ and NAD⁺-analogs was achieved by adding the right amount of stock solution of the cofactor in buffer in the 1 ml quartz semi-micro cuvette and bringing the volume up to 1 ml with buffer.

Isopropanol (10 μ l) was added to bring the substrate concentration to 100 mM.

Cuvette contents were stirred, enzyme solution was added (10 μ l), contents were stirred again and the reaction rate was monitored at the appropriate wavelength (section 2.4.2.c).

All assays were performed at a temperature of 25°C. In the cuvette cells such temperature was maintained by water circulating at 25°C. Substrate solutions and buffers were kept during the experiment in a 25°C water bath.

- (b) Michaelis constants of ADH alleloenzymes for isopropanol at pH values 4.85 - 7.45

Isopropanol (or Propan-2-ol, Analar grade, BDH Chemicals) was employed as the alcohol substrate and its Michaelis constant was estimated at several pH values (4.85-7.45 for S; 4.85-6.9 for F; 6.14-6.9 for F'). The range of solutions employed at each pH was based on preliminary experimental evidence. But both preliminary evidence and subsequent results were complicated by the phenomenon of substrate activation. Measurements that were affected by this phenomenon were not involved in the final calculation of the kinetic constants.

The Miller and Colder (1950) buffer system of constant ionic strength (0.1) was employed (Table 2.3).

- (c) Michaelis constants for NAD^+ and NAD^+ -analogs

These were estimated at pH 7.5 (buffer shown in Table 2.3). The NAD^+ used for these experiments was repurified according to Dalziel (1965) from commercial preparations. NAD^+ was dissolved in 0.2 M phosphate buffer pH 6.0 and the solution was passed through a Whatman DEAE-11 cellulose column. The column retains the NAD^+ , which is later eluted by a buffer concentration gradient (0.2 M - 1.5×10^{-3} M). /

Table 2.3 : Constant ionic strength (0.1) buffer solutions of Miller and Golder (1950)

pH*	5.0 M NaCl	2.0 M sodium acetate	8.5 M acetic acid	0.5 M Na_2HPO_4	4.0 M NaH_2PO_4
5.0 † (4.85)	32 ml	20.0 ml	3.7 ml		
5.5 † (5.35)	32 ml	20.0 ml	1.2 ml		
6.0 † (6.14)	32 ml			9.2 ml	6.6 ml
6.5 † (6.55)	32 ml			16.6 ml	3.7 ml
7.0 † (6.9)	32 ml			22.7 ml	1.6 ml
7.5 † (7.45)	32 ml			24.3 ml	0.5 ml

* Solutions must be made up to 2 l to give indicated pH

† Actual pH achieved in the experimental conditions reported here.

($0.2 \text{ M} - 1.5 \times 10^{-3} \text{ M}$). NAD^+ containing fractions are assayed spectrophotometrically and enzymatically with ADH which remains inactive in the presence of the contaminants of commercial NAD^+ preparations. These contaminants act as inhibitors (Dalziel, 1965) and if they are not removed can lead to erroneous results.

The two NAD^+ -analogs employed for the study of the enzyme cofactor interactions were:

(a) 3-acetyl-pyridine-adenine dinucleotide

(b) Thio-nicotinamide adenine dinucleotide

Throughout NAD^+ and NAD^+ -analog kinetics, isopropanol was used as the alcohol substrate. Concentrations (100 mM) were substantially higher than the recorded $K_m^{\text{isopropanol}}$ (app) at such pH.

Reaction rates for thio- NAD^+ were followed by the increase in absorption at 390 nm, and for 3-acetyl-PyAD at 365 nm.

(d) Calculation of maximal rates

Maximal rates (V_{max} or k_{cat}) per mole sub-unit were calculated with the use of accurate protein concentration estimates provided by amino acid analysis. Since the ADH-5 preparations used were of high purity (100%) and the amino acid composition is known, amino acid analysis provides a better tool for protein concentration estimation than either the Lowry or the modified Bradford methods. Amino acid analysis of samples was performed by the Rank-Hilger amino acid analyser and, in order to avoid the errors /

errors inherent in its integration procedure, non overlapping peaks were used for the estimation of the protein content of the samples (Lysine concentrations were usually employed).

(e) Computation and presentation of the results

Apparent Michaelis constants ($K_m(\text{app})$) and maximal rates were calculated from initial velocities by the ICL 2900 computer system of the Edinburgh Regional Computing Centre. The program used was written by Thatcher (1981, unpublished).

The program incorporates a sum of squares minimisation NAG routine (E04GEF) that was used to calculate the Michaelis constants. The routines of the program calculated the value of initial velocity (v_i) at given values of K_m and k_{cat} . This value was compared with the actual measured value of v_i (the residual) and new values for the Michaelis constants were generated by a Gaus-Newton algorithm which utilises the first derivative values of each constant. The minimum was usually reached within 50 cycles of calculation. The program is sophisticated in that discontinuities in the first derivative values of the Michaelis-Menten equation do not trap the interaction in local minima (an inverse Jacobean matrix is also used to check minimisation) and all calculations are performed in double precision arithmetic.

The results are presented as linear Lineweaver-Burke plots where the intercepts are the computer provided solutions to the Michaelis-Menten equation (Appendix II).

2.4.3 Thermal denaturation kinetics of ADH-F'-5 /

2.4.3 Thermal denaturation kinetics of ADH-F'-5

The experiment was performed in a Pye-Unicam SP-1800 split beam spectrophotometer fitted with an SP876 temperature programme controller. Temperature increase and absorbance change at 280 nm were monitored simultaneously using a W+W Tarkan 600 two channel recorder. Enzyme samples were diluted to a volume of 1 ml in a quartz semimicro cuvette with 0.1 M sodium phosphate buffer pH 6.0. A linear gradient of increasing temperature at different rates was used to denature the enzyme. The temperature at which denaturation commenced (signalled by the increase in absorbance at 280 nm) was taken as the "melting" temperature.

CHAPTER 3 Structural studies on ADH variants (F' and US)

3.1 Structural studies on the ADH-F' enzyme

3.1.1 Purification

The purification of this ADH variant followed the general procedures discussed in section 2.2. In a typical large experiment 1,700 g of larvae were used as starting material.

Some additions to the generalised purification procedure became necessary because of the amount and the nature of the starting material (Table 3.1). Sephadex G-25 desalting was coupled with dialysis in order to affect the drop in conductivity essential for the success of the ion-exchange steps. The Sephadex G-100 F gel filtration step was repeated, due to the volume of the sample in the first run that seriously affected the resolution of the ADH enzyme from the contaminant protease.

A serious problem, when using larvae as starting material, is melanin production in the homogenate which is mediated by the activity of a *Drosophila* phenyl oxidase, an enzyme that unfortunately remains in the ADH containing fraction until the final gel filtration step. However, it was found that its products are retained by DEAE-cellulose even in buffers with high ionic strengths. DEAE-52 cellulose chromatography was thus inserted twice in the purification routine /

Table 3.1 : Purification of ADH-F' from 1.7 kg of 3rd instar larvae

PURIFICATION STEP	VOLUME (ml)	PROTEIN (mg)	ACTIVITY (units)*	SPEC. ACTIVITY (units/mg)	LOSSES in activity between steps
Crude extract after prota- mine sulphate precipita- tion of nucleic acids	1,830	13,725	5,712	0.416	
0-40% ammonium sulphate saturation	1,940	12,210	5,364	0.439	6 %
40-60% ammonium sulphate saturation (after resu- sension of the preci- pitate)	172	7,610	4,161	0.546	22.5%
Sephadex G-25 F gel filtration	362	7,240	3,892	0.537	6.5%
Dialysis	390	6,825	3,774	0.552	3 %
After DEAE-52 cellulose and hydroxylapatite chro- matography	405	5,062	2,612	0.516	31 %
1st Sephadex G-100 F chromatography	157	805	1,668	2.072	36 %
2nd Sephadex G-100 F chromatography	67.5	345	1,005	2.913	40 %
2nd DEAE-52 cellulose chromatography	210	105	885	8.430	12 %
After freeze-drying		103	556	5.4	

* units are expressed in $\mu\text{mol NADH/min at } 4^{\circ}\text{C}$

routine (Table 3.1) achieving the removal of the pigments which seriously affect the performance of the hydroxyapatite column. In later preparations the phenyl oxidase was inhibited by the addition of thiourea (1 mM) in the buffers.

The purified enzyme contains all three isoenzymes which remain inseparable in this purification technique. Therefore the recorded specific activity is a composite one (Table 3.1) but, significantly, is less than half of the one recorded by Thatcher (1977) for ADH-UF, by a very similar technique, buffer and assay systems.

As can be seen in Table 3.1, hydroxyapatite chromatography and gel filtration are the steps where significant losses have occurred. A contributory factor for the severe losses during gel filtration has been the use of ammonium sulphate precipitation for the concentration of the sample before its application to the column. There has also been a sharp fall in activity after lyophilisation. This results probably from the partial denaturation of the protein during this step and it is further discussed later in this chapter.

3.1.2 Amino acid analysis of the ADH-F' enzyme

The purity of the prepared ADH-F' enzyme was tested by SDS-polyacrylamide electrophoresis. The sample containing the native enzyme revealed evidence of low protease contamination. Low molecular weight protein bands were present in its track that were missing from the track containing an oxidised sample of the preparation. /

Table 3.2 : Amino acid analysis of oxidized ADH-F'

Amino acid	(1)	(a1)	(b1)	(2)	(a2)	(b2)	(3)
Aspartic acid	199.771	27.9	(3.4%)	265.512	27.6	(4.8%)	29
Threonine	190.667	26.6	(8.3%)	247.120	25.7	(11.4%)	29
Serine	67.504	9.4	(4.4%)	87.402	9.1	(1.1%)	9
Glutamic acid	115.769	16.2	(4.7%)	139.004	14.5	(14.7%)	17
Proline	85.264	11.9	(8.2%)	119.882	12.5	(13.6%)	11
Glycine	138.590	19.3	(3.5%)	185.209	19.3	(3.5%)	20
Alanine	157.884	22	(4.5%)	211.526	22	(4.5%)	21
Valine	151.582	21.2	(3.6%)	214.634	22.3	(1.4%)	22
Isoleucine	145.574	20.3	(7.4%)	207.362	21.6	(6.0%)	23
Leucine	193.240	26.9	(0.4%)	240.398	25	(7.4%)	27
Tyrosine	42.091	5.9	(1.6%)	61.954	6.4	(6.6%)	6
Phenylalanine	61.517	8.6	(4.4%)	90.024	9.4	(4.4%)	9
Histidine	26.823	3.7	(7.5%)	59.520	6.7	(55.0%)*	4
Lysine	125.094	17.5	(2.9%)	166.745	17.4	(2.3%)	17
Arginine	41.841	5.8	(16.0%)+	60.129	6.3	(26.0%)+	5
Tryptophan		not determined					
Cysteine							
TOTAL		243.2			245.8		249
Average absolute deviation			4.2%			6.3%	

(1) & (2) 48 h analyses of ADH-F'.

(3) ADH-F composition as determined by Thatcher(1980) -nM/ml sample-.

(a) number of residues per mole estimated by adding the values for Asx, Glx, Pro, Gly, Ala, Leu, Phe, His, Lys, and Arg for each hydrolysate and dividing by 160 (the expected number of residues per mole for the above mentioned amino acids).

(b) deviation from the number of residues estimated from sequence information.

* † Very bad values excluded from calculation of average deviations.

preparation.

Since amino acid composition is known (Schwartz et al, 1975; Thatcher, 1977; Thatcher, 1980), amino acid analysis of the purified ADH-F' was performed as a more accurate assessment of purity, with the hope that it may also provide some evidence as to the identity of the residue (or residues) involved in the substitution.

Results from the amino acid analysis are shown in Table 3.2 and they are in good agreement with the ADH composition deduced from sequence information (Thatcher, 1980; Benyajati et al, 1981). The accuracy of the amino acid analysis for both hydrolysates is inside the limits set by Ambler (1981) of 3 to 8%.

These results verify the purity of the prepared ADH-F' but they do not provide any clues regarding possible substitutions.

3.1.3 Removal of protease traces - Random dissociation and reassociation of subunits - Bound protease?

As discussed before (section 2.2, 2.3), the protease that contaminates *Drosophila melanogaster* ADH preparations interferes adversely with the tryptic digestion of the protein as it attacks the C-terminus of several tryptic peptides (Thatcher, 1977) and so the removal of the small amounts present in the ADH-F' preparation has been necessary.

The major part of the lyophilised preparation was dissolved in 1 ml of 0.1 M Tris-HCl (pH 8.7) and passed through a smaller Sephadex /

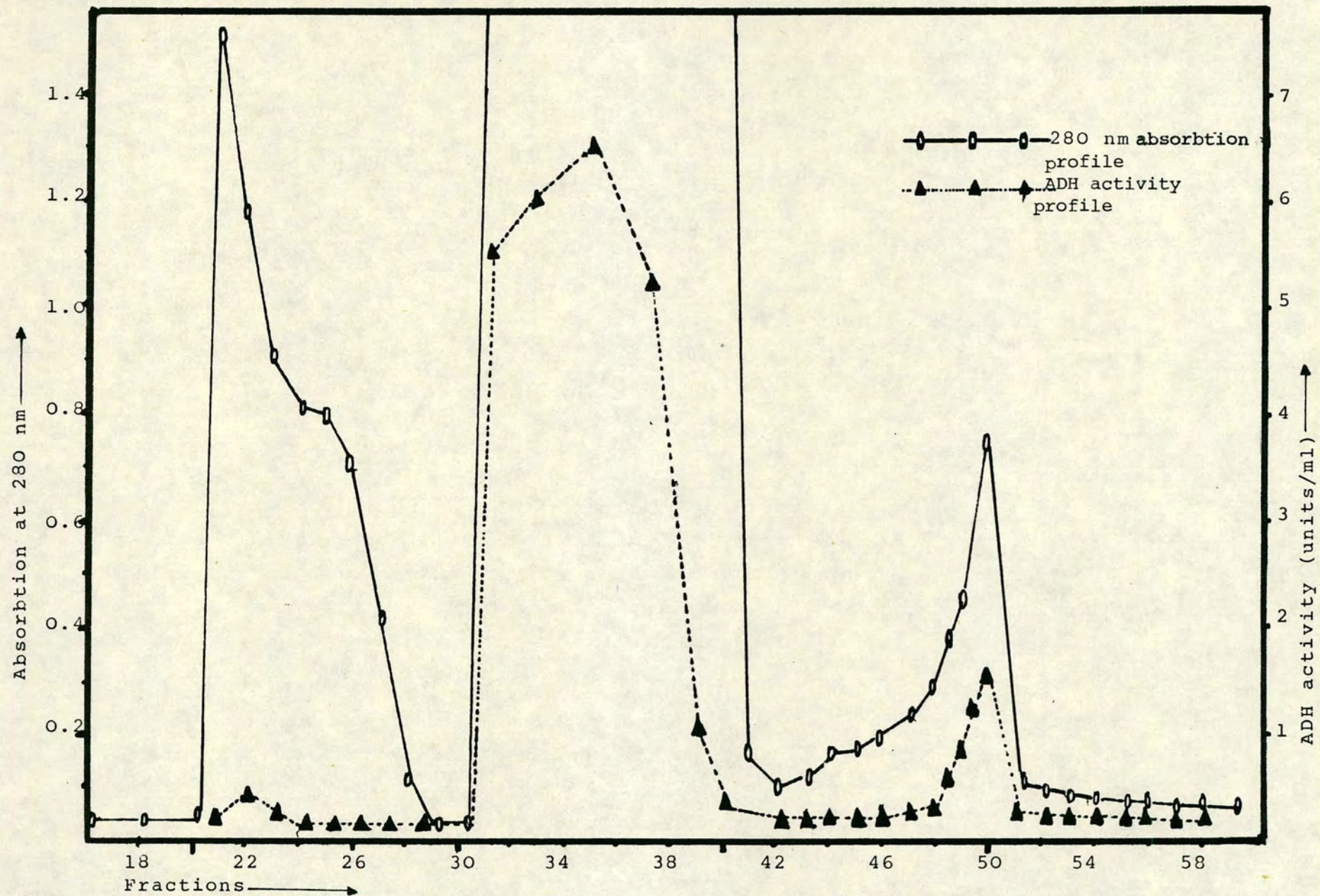


Fig. 3.1 . Sephadex G-100 F gel filtration of freeze-dried ADH-F'.

Sephadex G-100 F column (diameter 15 mm; length 750 mm). The results of the filtration (Fig. 3.1) show the presence of both tetramers and monomers of ADH in the preparation, an indication that some dissociation and reassociation of the subunits has taken place, either during lyophilisation or during resuspension of the freeze-dried protein.

There is also an asymmetric dispersion of activity in the native dimer peak. The 280 nm absorption to activity ratio showed that the trailing edge contained less activity than it ought to. This result along with the observed fall in specific activity observed after lyophilisation (section 3.1.1) leads to the assumption of partial inactivation occurring during freeze-drying or during resuspension of the solid.

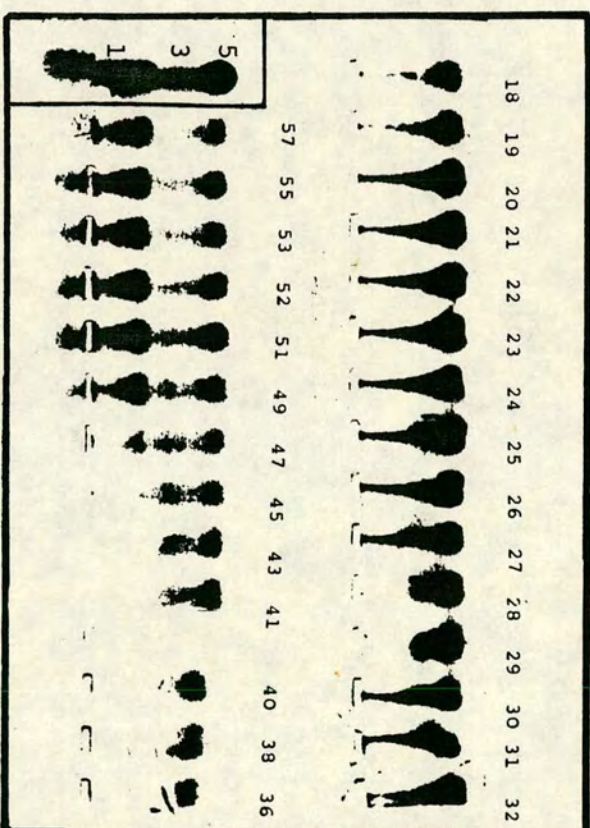
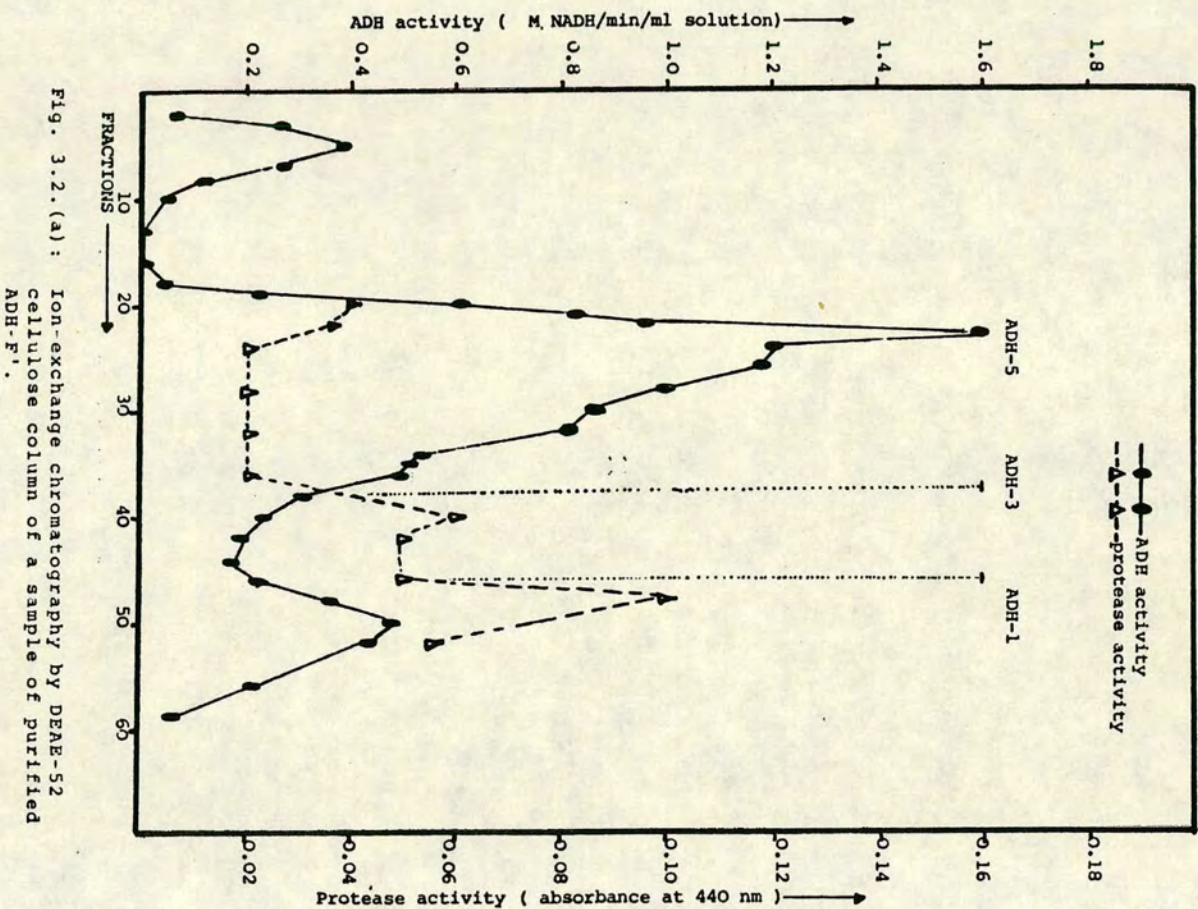
The activity detected both in the tetramer and monomer peaks might result from the spontaneous formation of the active dimer during the assay, and might not necessarily portray the enzymatic capabilities of these forms of the enzyme. The very low levels of activity associated with the tetramer peak probably signify that change in that direction is mostly irreversible.

It is probable that the partial inactivation and subunit dissociation and reassociation during lyophilisation are related phenomena, and could result from spontaneous chemical modification of residues involved in subunit interactions. Cysteine residues could be implicated. Thatcher (1981) showed that the chemical modification of a thiol group with 5,5'-dithio bis-(2 nitrobenzoic acid) partially inactivates the enzyme. Chemical interactions therefore /

therefore between cystine residues created during lyophilisation could explain the aggregate formation and the loss of reactivity.

The remaining part of the ADH-F' preparation was loaded on a DEAE-52 cellulose column (diameter 2.5 cm; length 10 cm) equilibrated with 0.075 M ammonium acetate buffer at pH 8.2. The enzyme was retained under such conditions. The column was then washed with a bed volume of buffer, that eluted some of the ADH. The rest of the enzyme was eluted with a shallow salt concentration gradient in the buffer (0 M - 0.03 M NaCl). Two peaks of activity were present when the elution fractions were assayed (Fig. 3.2.a). When aliquots of the fractions were electrophoresed in thin layer agarose gels (Fig. 3.2.b) it was found that the leading peak consisted purely of ADH-5. The trailing peak contained mostly ADH-1 but the small amounts of ADH-5 were visible because of the much higher specific activity of this isoenzyme. Small amounts of ADH-3 were present in the fractions eluted between the two major peaks.

The fractions were also assayed for protease content by the azoalbumin method described in section 2.2. As can be seen from Fig. 3.2.a the peaks of protease activity were associated with the appearance of the different isoenzymes in the elution profile. This behaviour, as well as the continuous contamination of ADH fractions after various fractionisations with several agents, leads to a hypothesis of a bound protease. The existence of an enzyme-bound protease has been postulated for hexokinase (Rustum et al, 1971) and yeast ADH (Grunow and Schöpp, 1978). If such a proposition is true for *Drosophila* ADH then most of the protease must be associated with /



with ADH-3 and ADH-1 isoenzymes.

3.1.4 Tryptic digestion - Fractionisation and mapping of tryptic peptides

50 mg (1,82 μ moles of ADH subunit) of freeze-dried ADH-F' were oxidised as described in section 2.3. Tryptic hydrolysis of the oxidised protein was then performed (as in section 2.3).

The tryptic digest was subsequently fractionated by Sephadex G-25 SF gel filtration and the fractions were electrophoresed on paper at pH 6.5 (section 2.3). Ninhydrin and subsequently Pauly staining were used to visualise the peptides.

Results are shown in Fig. 3.3. Tryptic peptide nomenclature is the one employed by Thatcher (1980). Their location in the sequence is shown in Table 3.3.

Since, as mentioned in section 2.3, a significant proportion (up to 60% of the sequence of ADH-F) of tryptic peptides are insoluble or of poor solubility, the "classical" chromatography-electrophoresis mapping technique does not provide a very effective tool for the quick spotting of substitutions (as for example is possible in human hemoglobin). But the "map" resulting from the filtration-electrophoresis sequence described above does supply significant information about substitutions in the soluble peptides resulting from enzymic digestion when coupled with the subsequent development of spots in BAWP chromatography and paper electrophoresis at different pHs.

The /

The gel filtration-electrophoresis map can easily distinguish between the S and F ADH mutants. As can be seen in Fig. 3.4 and Fig. 3.5 which show maps of ADH-S and ADH-F respectively, derived from similar experiments, the S digest exhibits two extra peptides: peptides T14a and T14b. They result from the extra tryptic cleavage position provided by the presence in the ADH-S enzyme of a lysine residue in position 192. The composite peptide T14 that is present in the ADH-F tryptic digests is of poor solubility and usually inaccessible to this mapping procedure.

Going back to the results obtained for ADH-F' (Fig. 3.3) it can be seen that the peptides T14a and T14b are present in the electrophoretogram. (T14a, containing a histidine residue, responds to Pauly staining, further facilitating recognition). This suggests that ADH-F' may be related to ADH-S in sequence rather than to ADH-F which it is like in electrophoretic mobility. Clues as to the substitution responsible for the ADH-F-like mobility were provided by the disappearance of the T6 peptide from its expected position among the basic tryptic peptides. It could be therefore assumed that the substitution affected either the charge of this peptide or the cleavage position between T6 and T7. (T7 is of poor solubility and not usually present in this kind of "map").

3.1.5 Purification of the soluble tryptic peptides - Discovery of the mutant peptide

Fractions 65 to 92, containing the soluble tryptic peptides (Fig. 3.3) /

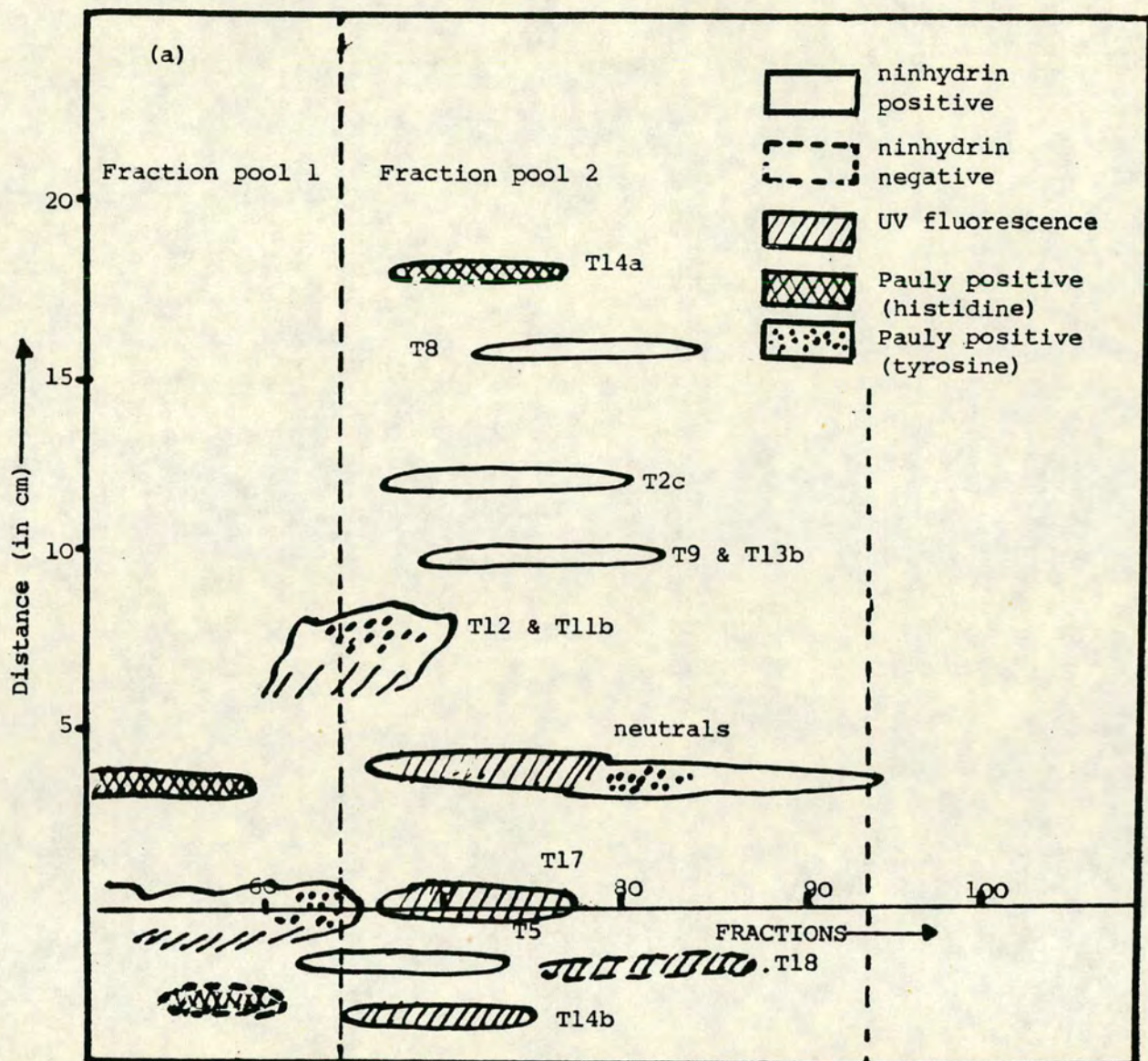
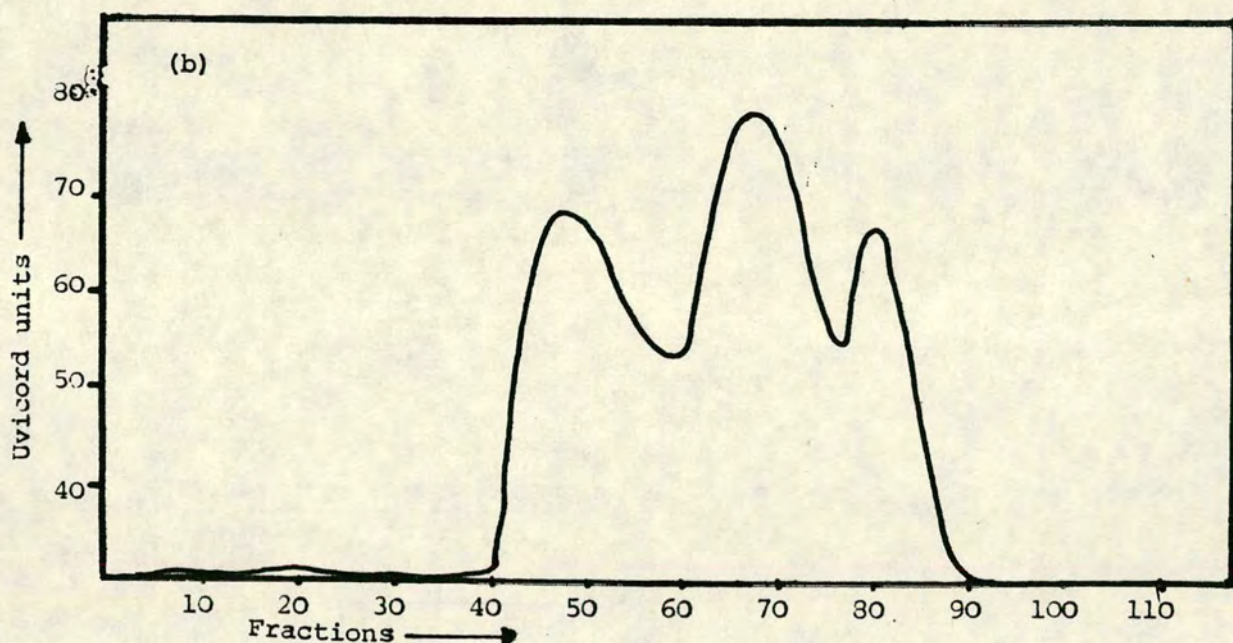


Fig. 3.3.(a): HVPE at pH 6.5 of the tryptic digest of oxidized *Drosophila* ADH-F' after fractionization by Sephadex G-25 SF filtration.

Fig. 3.3.(b): Sephadex G-25 SF elution profile of the ADH-F' digest.



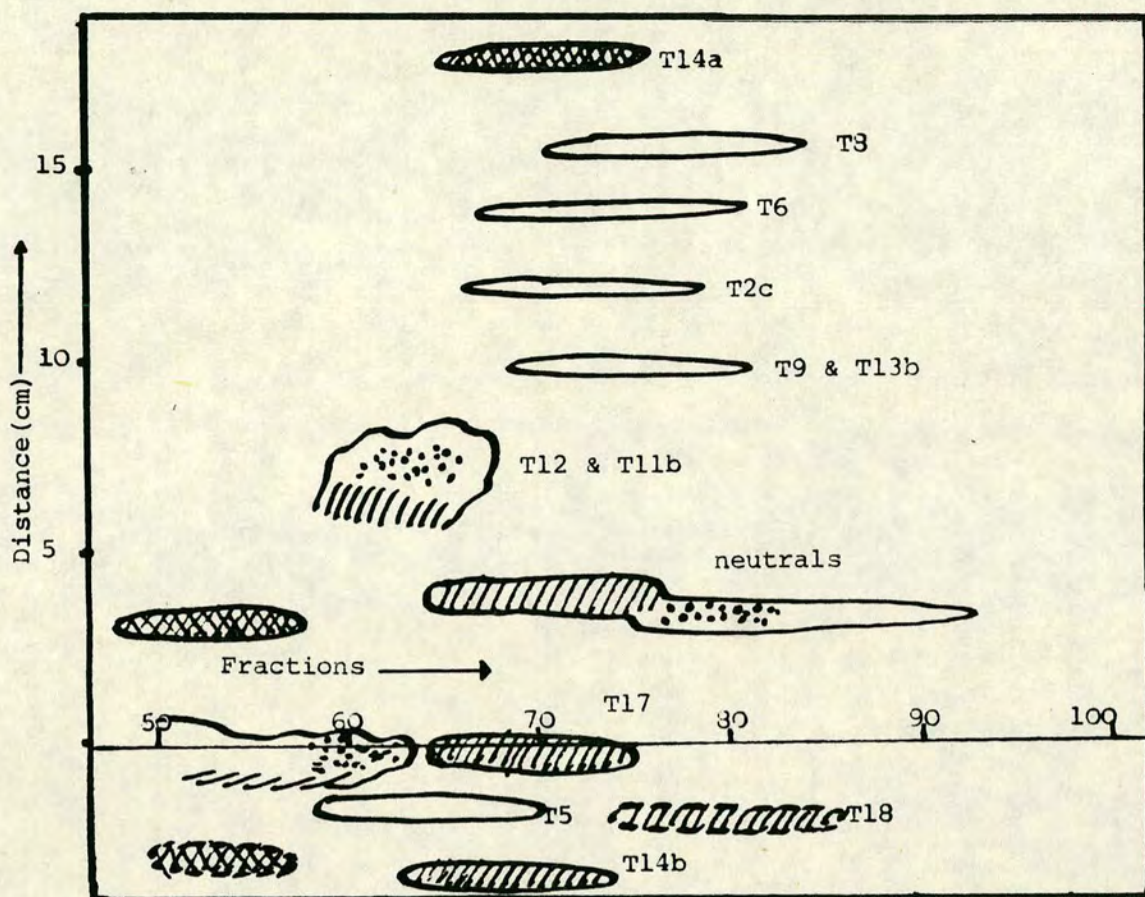


Fig. 3.4 : Typical HVPE at pH 6.5 of tryptic digests of oxidized ADH-S after fractionization by Sephadex G-25 SF gel filtration.

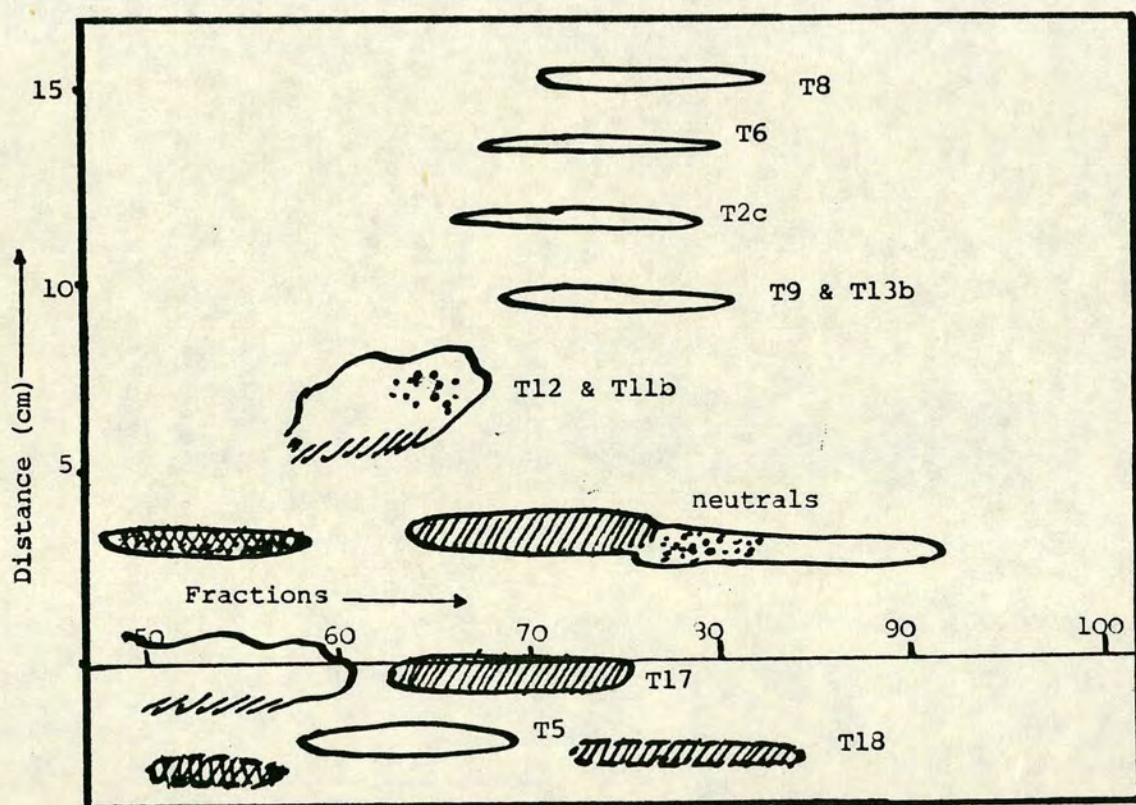


Fig. 3.5 : Typical HVPE at pH 6.5 of tryptic digests of oxidized ADH-F after fractionization by Sephadex G-25 SF gel filtration

Table 3.3 : Tryptic peptides of ADH-S †

T1	Acetyl- ¹ S-F-T-L-T-N-K
T2	N-V- ¹⁰ I-F-V-A-G-L-G-G-I-G- ²⁰ L-D-T-S-K (*)
T2c	E-L-L-K-R ; T2b E-L-L-K
T3	³⁰ D-L-K
T4	N-I-V-I-L-D-R
T5	⁴⁰ I-E-N-P-A-A-I-A-E-L- ⁵⁰ K
T6	A-I-N-P-K
T7	V-T-V-T- ⁶⁰ F-Y-P-Y-T-V-T-V-P-I- ⁷⁰ A-E-T-T-K (*)
T8	L-L-K
T9	T-I- ⁸⁰ F-A-Q-L-K
T10	T-V-D-V-L- ⁹⁰ I-N-G-A-G-I-L-D-D-H- ¹⁰⁰ Q-I-E-R (**) T-I-A-V-N-Y- ¹¹⁰ T-G-I-V-N-T-T-T-A-I- ¹²⁰ I-D-F-W-D-K , R , K (***)
T11	¹³⁰ G-G-P-G-G-I-I-C-N-I-G-(S, ¹⁴⁰ V,T,)-G-F-N-A-I-Y-Q-V- ¹⁵⁰ P-V-Y-S- -G-T-K (*)
T12	A-A-V- ¹⁶⁰ V-N-F-T-S-S-L-A-K (*)
T13	¹⁷⁰ L-A-P-I-T-G-V-T-A-Y-T- ¹⁸⁰ V-N-P-G-T-T-R (**) T14a T-T-L- ¹⁹⁰ V-H-K
T14b	F-N-S-W-L-D-V- ²⁰⁰ E-P-Q-V-A-E-K
T15	L-L-A- ²¹⁰ H-P-T-Q-P-S-L-A-C-A- ²²⁰ E-N-F-V-K (*)
T16	A-I-E-L-N- ²³⁰ Q-N-G-A-I-W-K
T17	L-D-L- ²⁴⁰ G-T-L-E-A-I-Q-W-T-K
T18	²⁵⁰ H-W-D-S-G- ²⁵⁵ I-COOH (*)

Pseudotryptic peptides usually found in tryptic digests

T12a	A-A-V-V-N-F ; T12b T-S-S-L-A-K
T13a	L-A-P-I-T-G-V-T-A-Y ; T13b T-V-N-P-G-T-T-R
T11b	N-A-I-Y-Q-V-P-V-Y-S-G-T-K

† Thatcher (1980)

(*), (**), (***) degrees of insolubility

(Fig. 3.3) were pooled and freeze-dried. The sample was resuspended in 1 ml of 6% glacial acetic acid. The peptides were then separated by preparative paper electrophoresis and elution (section 2.3). They were dried in vacuo and redissolved in 0.25 ml of N/10 ammonia. T17 and T5 peptides ran too close together and were therefore eluted as a single spot. Their separation was left for subsequent steps.

The isolated peptides were electrophoresed at pH 3.5 and chromatographed in paper in BAWP developing medium (section 2.3). Results are shown in Fig. 3.6 and Fig. 3.7. As can be seen, peptides T17 and T5 were easily separable by both techniques.

The weakly basic peptide T12 was shown to contain a contaminant peptide, separable in HVPE at pH 3.5, being in this buffer slightly less positively charged than T12. (In subsequent stages this peptide was shown to be the "pseudotryptic" T11b).

T9 and T13b peptides showed almost identical electrophoretic mobilities both in pH 3.5 and 6.5 (Fig. 3.6). Good separation was only achieved through the BAWP medium.

Peptides T16, T17 and T14b displayed fluorescence under UV irradiation, as expected, because of the tryptophan residue they contain (Table 3.3). T16 displays three distinct electrophoretic forms in HVPE at pH 3.5, observed also by Thatcher (1980). The basis of this phenomenon might be due to the different oxidation states of the tryptophan side chain.

The position on paper of peptide T18 was designated purely by /

by its fluorescence under UV lighting, since this peptide is almost ninhydrin negative. Although UV fluorescence usually indicates the presence of tryptophan residues, Thatcher (1980) failed to detect the presence of such a residue in the peptide by manual dansyl-Edman degradation sequencing. Benyajati et al (1981) confirmed its existence by DNA sequencing of the gene in position 251.

The neutral peptides though, as separated by BAWP chromatography, provided some answers as to the nature and position of the substitution. All expected neutral soluble peptides were present (as can be seen by comparison with the ones present in the neutral band after tryptic digestion of ADH-F shown in Fig. 3.7). An additional spot though was also noted, slower than the rest, termed T6-F'.

All peptides were subsequently purified through the preparative versions of the techniques mentioned above.

3.1.6 N-terminus determination, qualitative and quantitative amino acid analysis, sequential degradation of soluble tryptic peptides of ADH-F'

N-terminal amino acid analysis (performed as described in section 2.3) was used to establish the identity of the peptides in the sequence and to show that they were adequately pure. Qualitative and quantitative amino acid analysis and sequential degradation were then performed (Results are shown in Tables 3.4, 3.5, 3.6).

At this stage the presence of "pseudotryptic" peptides (Keil-Dlouha et al, 1971) T13a, T13b and T11b was confirmed. They result from secondary cleavages after aromatic amino acid residues (phenylalanine and tyrosine only in this case because tryptophan residues have been affected by the oxidation), and are commonly encountered in tryptic digests of ADH (Thatcher, 1980).

The quantitative amino acid analysis of the peptide T13a did not reveal the presence of tyrosine. Instead an unidentified peak was present in the 590 nm absorption recording, eluting just before histidine. This peak probably represents chloro-tyrosine, produced under oxidising conditions in the presence of NaCl (Sanger and Thomson, 1953). However, the tyrosine residue was detected in the qualitative amino acid analysis and Dns-o-tyrosine (strong yellow spot under UV light) was present on the polyamide sheets during N-terminus determination and in all stages of sequence determination (tyrosine being the C-terminal residue).

The amino acid analysis of peptide T11b also showed smaller amounts of tyrosine than expected from the sequence (Table 3.3). During sequential degradation of this peptide the removal of the first three (3) residues resulted in a peptide of poor solubility. This, in conjunction with the small amounts available, prevented further sequencing. Its electrophoretic mobility though and its amino acid composition (close to the one deduced from the sequence) suggest that it is identical to the one recovered and sequenced by Thatcher (1980).

Surprisingly, /

Surprisingly, another set of common "pseudotryptic" peptides, Tl2a (A-A-V-V-N-F, poor solubility) and Tl2b (T-S-S-L-A-K, showing identical electrophoretic mobility at pH 6.5 with Tl4a), were not encountered although the Tl2b fragment is of good solubility. It can be assumed that the conditions of digestion did not favour their formation in this case. Intact Tl2 was recovered in small yields.

Amino acid analysis of Tl2 (Table 3.5) showed the presence of only one valine residue against the two expected. Acid hydrolysis had only been for the standard 24 hours and the low valine yields in this case can be explained by the particular resistance of the Val-Val peptide bond to acid hydrolysis. Small amounts and poor solubility made sequential degradation rather difficult and only three cycles were completed.

Peptide Tl8 gave a substandard analysis. Although both published sequences (Thatcher, 1980; Benyajati et al, 1981) give histidine as the N-terminus of the peptide, there was no reaction with the Pauly reagent. No N-terminus was determined after dansylation and sequential degradation proved impossible. As mentioned above it is also ninhydrin negative, its detection on paper depending upon fluorescence under UV light. But qualitative amino acid analysis showed that the peptide had the expected complement of amino acids and from its behaviour in electrophoresis it can be safely assumed that this peptide is unaltered in ADH-F'.

The N-terminus determination of the mutant peptide T6-F' showed /

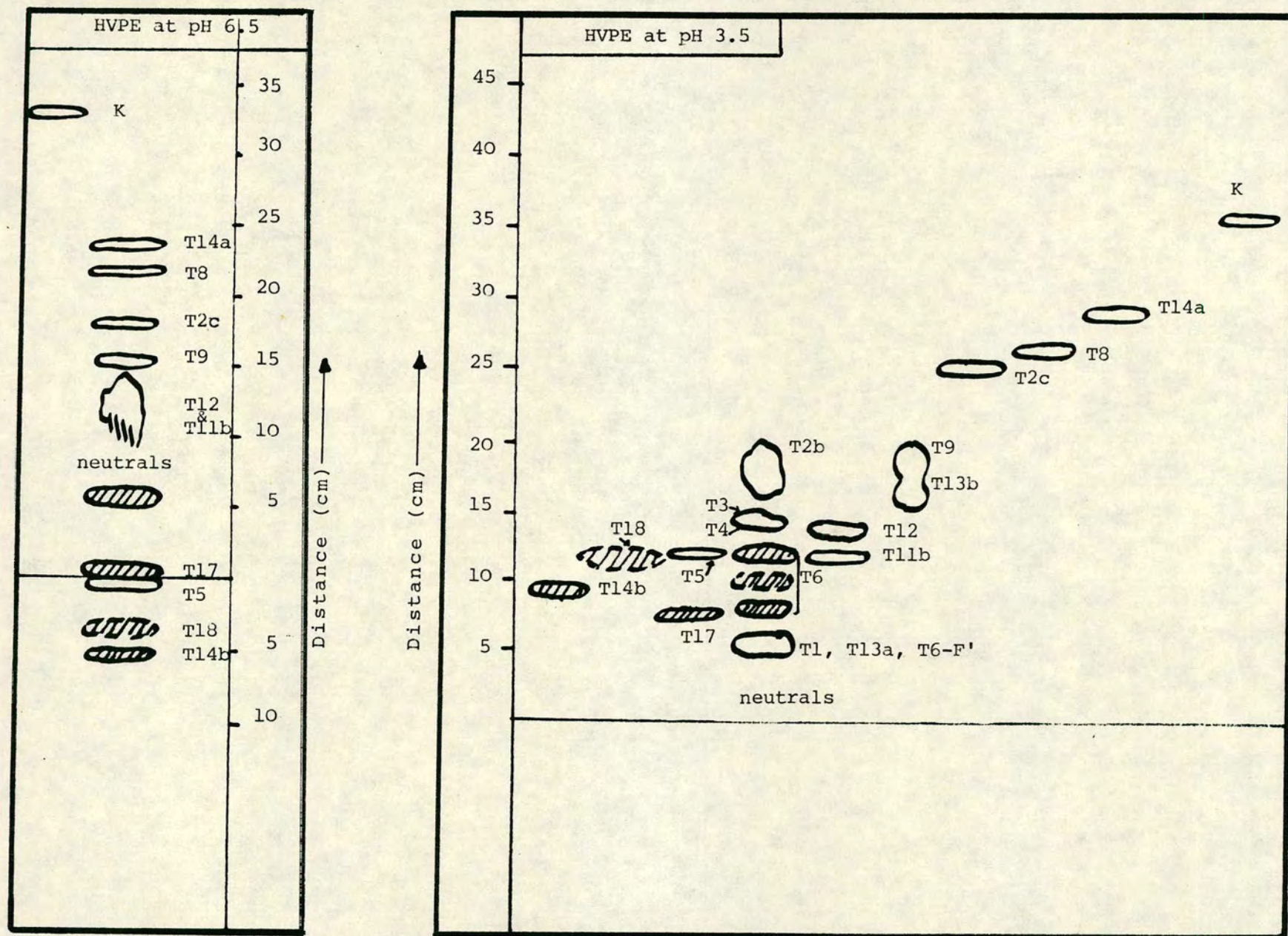


Fig. 3.6 : HVPE at pH 6.5 and 3.5 of soluble tryptic peptides of ADH-F'

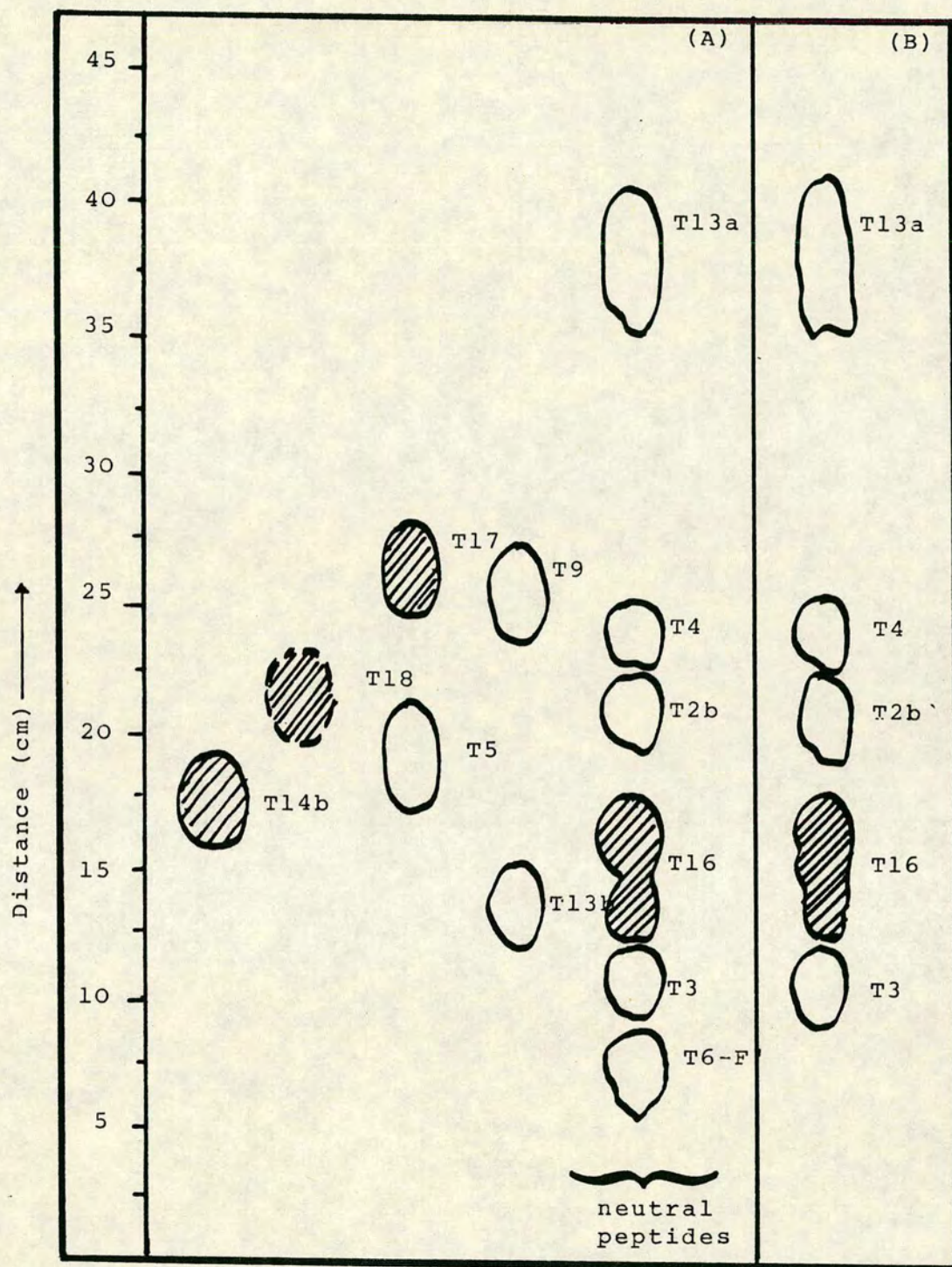


Fig. 3.7 : BAWP chromatography of tryptic peptides of oxidized ADH-F' (column B shows the separation of the neutral peptides from a tryptic digest of ADH-F).

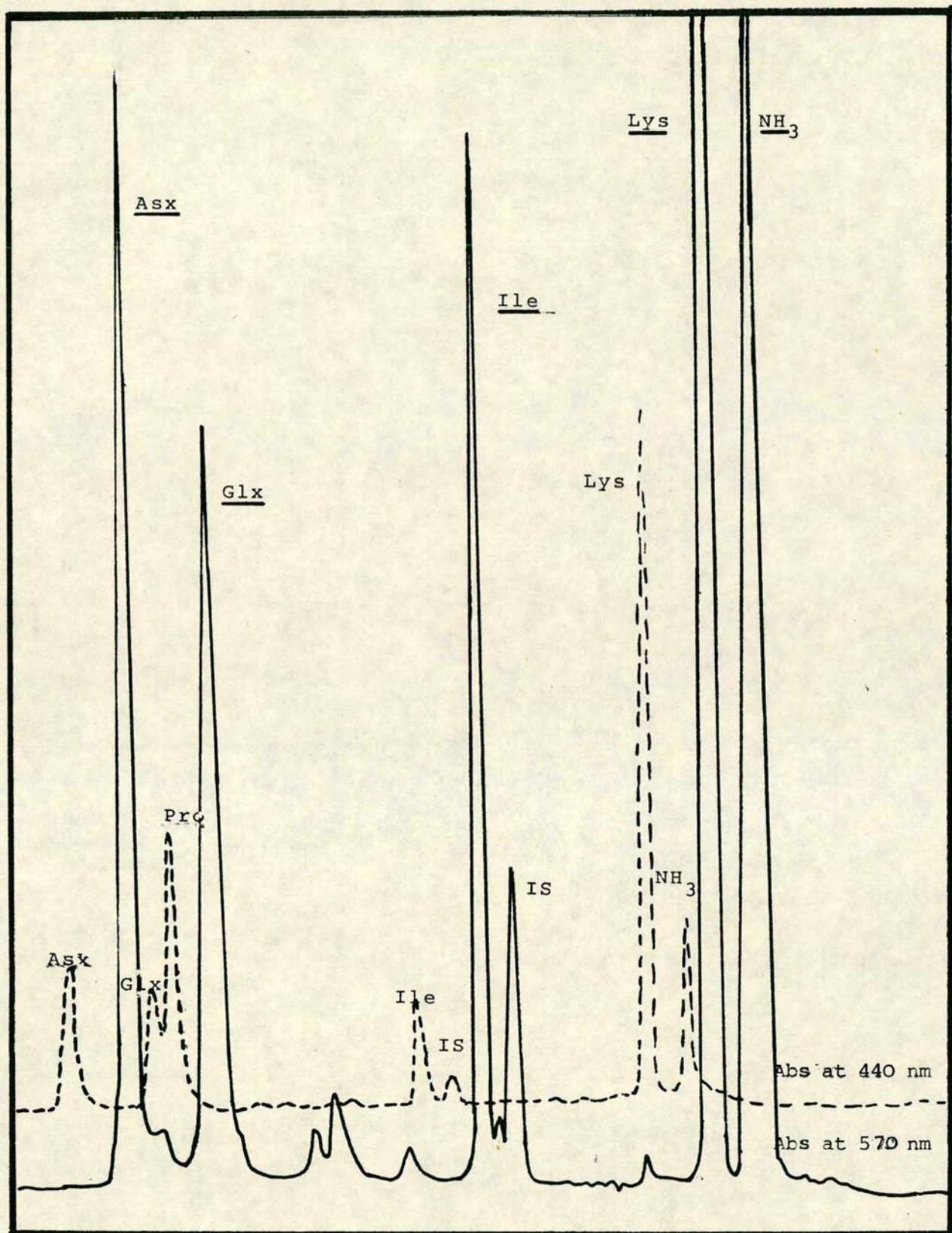


Fig. 3.8 : Quantitative amino acid analysis of peptide T6-F'.
(IS = internal standard)

Table 3.4 : Electrophoretic mobility, N-terminus and qualitative amino acid analysis of the tryptic peptides of ADH-F'

Peptide		electrophoretic mobility		N-terminus	qualitative amino acid analysis
		pH 6.5	pH 3.5		
T14a	basic	0.65	0.88	Thr	K, H, L, V, T
T8	"	0.56	0.76	Leu	K, L
T2c	"	0.42	0.74	Glx	K, R, L, E
T9	"	0.32	0.54	Thr	K, A, L(?), T, E, F
T13b	"	0.32	0.53	Thr	R, G, V(?), P, T, D
T12	"	0.24	0.42	Ala	K, A, V, L, S(?), T, F, D
T11b	"	0.24	0.39	Asx	K, G, A, V(?), I, S, P, T, Y, D, E
T6-F'	neutral	0	0.53	Glx	K, I(?), P, D, E
T3	"	0	0.44	Asx	K, L, D
T16 *	"	0	0.24 (0.27) (0.33)	Ala	K, G, A, L, I(?), D, E
T2b	"	0	0.55	Glx	K, L, E
T4	"	0	0.23	lost	lost
T13a	"	0	0.23	Leu	G, A, L, V, I(?), P, T, Y
T17	acidic	-0.18	0.26	Leu	K, G, A, L, I(?), T, D, E
T5	"	-0.22	0.38	Ile	K, A, L, I, P, D, E
T14b	"	-0.27	0.27	Phe	K, G, A, V, I(?), S(?), P, F, D, E
T18	"	-0.33	0.39	-	G, I(?), S(?), D

* This peptide presented three electrophoretic forms in HVPE at pH 3.5
(?) dubious identification due to the proximity of spots and overloading

Table 3.5 : Quantitative amino acid analysis of soluble tryptic peptides of ADH-F'

Amino acid peptide	(%)	Recov.	Asx	Thr	Ser	Glx	Pro	Gly	Ala	Val	Ile	Leu	Tyr	Phe	His	Lys	Arg
T14a	(a) 4.1			2.14						0.91		1.03			0.99	1.10	
	(b)			2						1		1			1	1	
T8	(a) 9.9											1.91				1.08	
	(b)											2				1	
T2c	(a) 6.7					0.95						2.18				0.94	0.91
	(b)					1						2				1	1
T13b	(a) 12.5	1.10	2.06				1.10	1.21		0.83	0.85						0.82
	(b)	1	2				1	1		1	1						1
T9	(a) 1.5		1.01			0.93			1.04		0.74	1.15		1.04		1.08	
	(b)		1			1			1		1	1		1		1	
T12	(a) 0.7	1.26	1.50	1.99					2.43	0.81		1.05		0.77		0.97	
	(b)	1	1	2					3	1		1		1		1	
T11b	(a) 2.2	0.96	1.02	1.17	0.96	1.02	0.83	1.18	1.44	0.40			1.04			0.82	
	(b)	1	1	1	1	1	1	1	2	1			2			1	
T6-F	(a) 11.4	0.98				0.91	1.07					0.95				1.09	
	(b)	1				1	1					1				1	

continued/

Table 3.5 : (continued)

peptide	Amino acid	Recov. (%)	Asx	Thr	Ser	Glx	Pro	Gly	Ala	Val	Ile	Leu	Tyr	Phe	His	Lys	Arg
T3	(a)	5.2	1.32									0.83				0.85	
	(b)		1									1				1	
T2b	(a)	6.5				0.98						1.35				0.78	
	(b)					1						2				1	
T16	(a)	2.3	2.62			1.57		1.42	1.55		1.40	1.23				1.20	
	(b)		2			2		1	2		2	1				1	
T13a	(a)	11.1		0.80			1.22	1.14	1.95	0.83	0.96	0.88	-				
	(b)			1			1	1	2	1	1	1	1				
T17	(a)	5.5	1.67	2.64		1.98		1.09	1.25		1.01	2.60				0.82	
	(b)		1	2		2		1	1		1	2				1	
T5	(a)	6.1	1.40			1.98	1.30		2.54		1.69	1.15				0.94	
	(b)		1			2	1		3		2	1				1	
T14b	(a)	8.3	3.11		0.88	2.40	1.11		0.89	1.38		1.05		0.80		0.74	
	(b)		2		1	3	1		1	2		1		1		1	

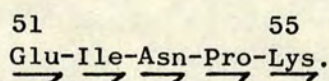
(a) : residues / mole peptide calculated from analysis results.

(b) : composition deduced from sequence information.

Table 3.6 : Extent of sequencing of soluble tryptic peptides of ADH-F'

Peptide	Total number of residues	N ^o of residue										remaining residues
		N-term.	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	
T2c	5	E	L	L	K	R						
T2b	4	E	L	L	K							
T3	3	D	L	K								
T5	11	I	E	N	P	A	A	I	A		-(E, L)-K
T6-F'	5	E	I	N	P	K						
T8	3	L	L	K								
T9	7	T	I	F	A	Q	L	K				
T12	12	A	A	V							-(V, N, F, T, S, S, L, A)-K
T14a	6	T	T	L	V	H	K					
T14b	14	F	N	S	(W)	L	D	V			-(E, P, Q, V, A, E)-K
T16	12	A	I	E	L	N	Q	N	G		-(A, I, W)-K
T17	13	L	D	L	G	T	L	E	A	I	-(Q, W, T)-K
T13a	10	L	A	P	I	T	G	V	T	A	Y	
T13b	8	T	V	N	P	G	T	T	R			
T11b	13	N	A	I	Y						-(Q, V, P, V, T, S, G, T)-K

showed the presence of Dns-Glu against the expected Dns-Ala (alanine is the residue of position 51 in the sequence of all ADH variants investigated so far). During sequential degradation after the removal of the first two residues (Glx and Ile) the remaining peptide had a positive charge in HVPE in pH 6.5, thus identifying positively asparagine as the residue occupying position 53. Since the whole peptide is electrophoretically neutral at pH 6.5, glutamic acid has therefore substituted alanine in position 51 of the sequence of ADH-F' enzyme. The full sequence of peptide T6-F' reads as follows:



The rest of the recovered tryptic peptides (T14a, T14b, T2c, T2b, T8, T9, T13b, T16, T5, T17) showed no deviation from the expected sequence. Electrophoretic mobility, amino acid composition and sequential degradation showed them to be indistinguishable from the ones recovered and sequenced by Thatcher (1980).

3.1.7 Sequence information from high molecular weight peptides of poor solubility obtained from tryptic digestion of ADH-F'

3.1.7.a Strategies employed

Fractions 45-64 (Fig. 3.3) were pooled and freeze-dried. The peptides in this fraction are strongly hydrophobic and cannot be purified by methods that employ paper as the supporting phase (only peptide T17 responds favourably to such techniques). However, they can be purified by DEAE or CM-cellulose chromatography in urea buffers, /

buffers, but as Thatcher (1980) notices, even these methods are not entirely satisfactory. Results are usually not reproducible and separation in many cases incomplete. Significant losses should also be expected for the most hydrophobic peptides. Complications also arise from the fact that small changes in urea concentrations interfere unfavourably with the performance of the resin. On the other hand chymotryptic or thermolytic digestion of the total fraction results in a multiplicity of peptides of low molecular weight. A combination of these methods achieves a survey of at least 60% of the sequence incorporated in the hydrophobic tryptic peptides.

3.1.7.b Ion-exchange chromatography in urea of hydrophobic tryptic peptides

Half of the freeze-dried fraction was dissolved in 4M urea in 0.015 M ammonium acetate pH 7.5 and added to a DEAE-52-cellulose column (70 mm in length, 10 mm in diameter), equilibrated with the same buffer. After addition, the column was washed with two bed volumes of buffer and subsequently a salt concentration gradient was applied (0 M - 0.3 M NaCl).

The elution profile showed four peaks (Fig. 3.9). Fractions containing each peak were pooled and desalted with the use of a Sephadex G-15 column (length 150 mm, diameter 15 mm) equilibrated in 1 M acetic . The desalted fractions were then freeze-dried.

The /

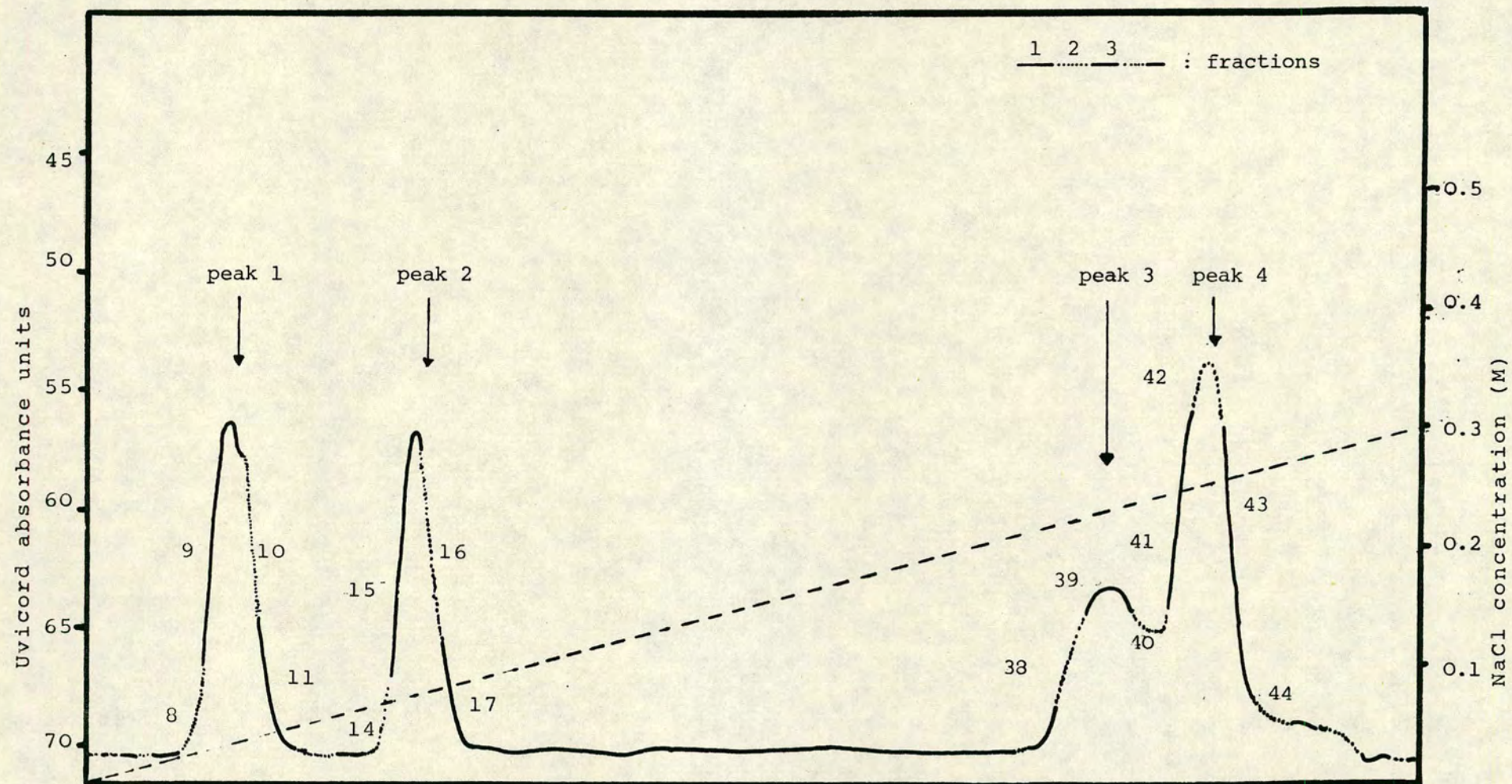


Fig. 3.9 : DEAE-52 cellulose ion-exchange chromatography of fraction pool 1 that contains high molecular weight peptides of poor solubility.

The N-terminus of the contents of each peak was determined to help identify the eluted peptides and assess their degree of purity. After the third dimension the following spots were visible on polyamide sheets (apart from basic Dns-amino acids).

Peak 1: Dns-Phe, Dns-Gly, Dns-Thr

Peak 2: Dns-Asx, Dns-Gly

Peak 3: Dns-Leu

Peak 4: Dns-Val, Dns-o-Tyr

On the basis of these results peaks 1 and 2 are impure, being mixtures of at least two peptides (peak 1 probably contains remnants of T14b and peptide T10 while peak 2 is a mixture of peptides T2 and T11a). Further purification of these peptides was considered unproductive because of the small amounts recovered.

Peak 3 displaying Leucine as N-terminus could be the tryptic peptide T15, the only insoluble tryptic peptide expected to possess such an N-terminal residue.

Peak 4: Valine at the N-terminus and the presence of good amounts of Dns-o-tyrosine on the polyamide sheets provisionally identified the contents of peak 4 as the tryptic peptide T7.

3.1.7.b(1) Thermolytic digestion of insoluble tryptic peptides

In order to establish the identity of the purified peptides and to determine their sequences they were digested with thermolysin as described in section 2.3. Thermolysin was preferred to chymo-
:trypsin

chymotrypsin because the expected thermolytic peptides cover most of the sequence and are easier to handle. This is especially true for peptide T15 where there is only one primary chymotryptic cleavage site very close to the C-terminus and the chymotryptic peptide that can be recovered is only three residues shorter than the tryptic one. Also the recoverable chymotryptic peptides after digestion of T7 cover only 60% of the sequence (Thatcher, 1980). Thermolytic digestion of T7 had not been attempted by Thatcher (1980), so no guide existed as to the pattern of peptides expected, though some thermolytic (H13, H15) peptides, parts of the T7 sequence, were recovered by the aforementioned author, after thermolytic digestion of the whole protein.

After desalting by Sephadex G-15 column (150 mm length, 15 mm diameter) the products of digestion were electrophoresed on paper. Figure 3.9 shows the thermolytic peptides present of both T7 and T15 after HVPE at pH 6, 5 and pH 3.5.

H3b1 (Pauly positive for histidine), H3b2, H3n, H3a were the thermolytic peptides isolated from the peak 3 tryptic peptide (T15 ?).

The ones isolated for the tryptic peptide of peak 4 (T7 ?) were: H4b, H4n, H4a1, H4a2 (H4a2 was Pauly positive for tyrosine).

Their purity was established by N-terminus determination and both qualitative and quantitative amino acid analyses were performed.

3.1.7.b(2) ADH-F' T15 thermolytic peptides

The whole of the T15 sequence was contained in the isolated thermolytic peptides.

H3b2: amino acid analysis (Table 3.8) showed only valine and lysine present, in equimolar amounts. Since valine is the N-terminus (Table 3.7), the sequence is: V-K.

H3n: both amino acid analysis and N-terminus determination showed it to be free leucine.

Amino acid analysis and N-terminus determination identified H3b1 and H3a with T15Ha and T15Hb purified by Thatcher (1980) after thermolytic digestion of T15. Sequential degradation of these peptides verified the identification:

H3b1: L-A-H-P-T-Q-(P, S)
 $\xrightarrow{\quad} \xrightarrow{\quad} \xrightarrow{\quad} \xrightarrow{\quad} \xrightarrow{\quad}$

H3a: L-A-C-A-E-N-F
 $\xrightarrow{\quad} \xrightarrow{\quad} \xrightarrow{\quad} \xrightarrow{\quad} \xrightarrow{\quad}$

From the thermolytic peptides the sequence of ADH-F' T15 can be pieced together with the help of the known sequence:

L-L-A-H-P-T-Q-P-S-L-A-C-E-N-F-V-K
 $\uparrow \quad \uparrow \quad \quad \uparrow \quad \quad \uparrow$
 H3b H3b1 H3a H3b2

Therefore ADH-F' T15 is identical with the T15 present in ADH-F and S (Table 3.3).

3.1.7.b(3) ADH-F' T7 thermolytic peptides /

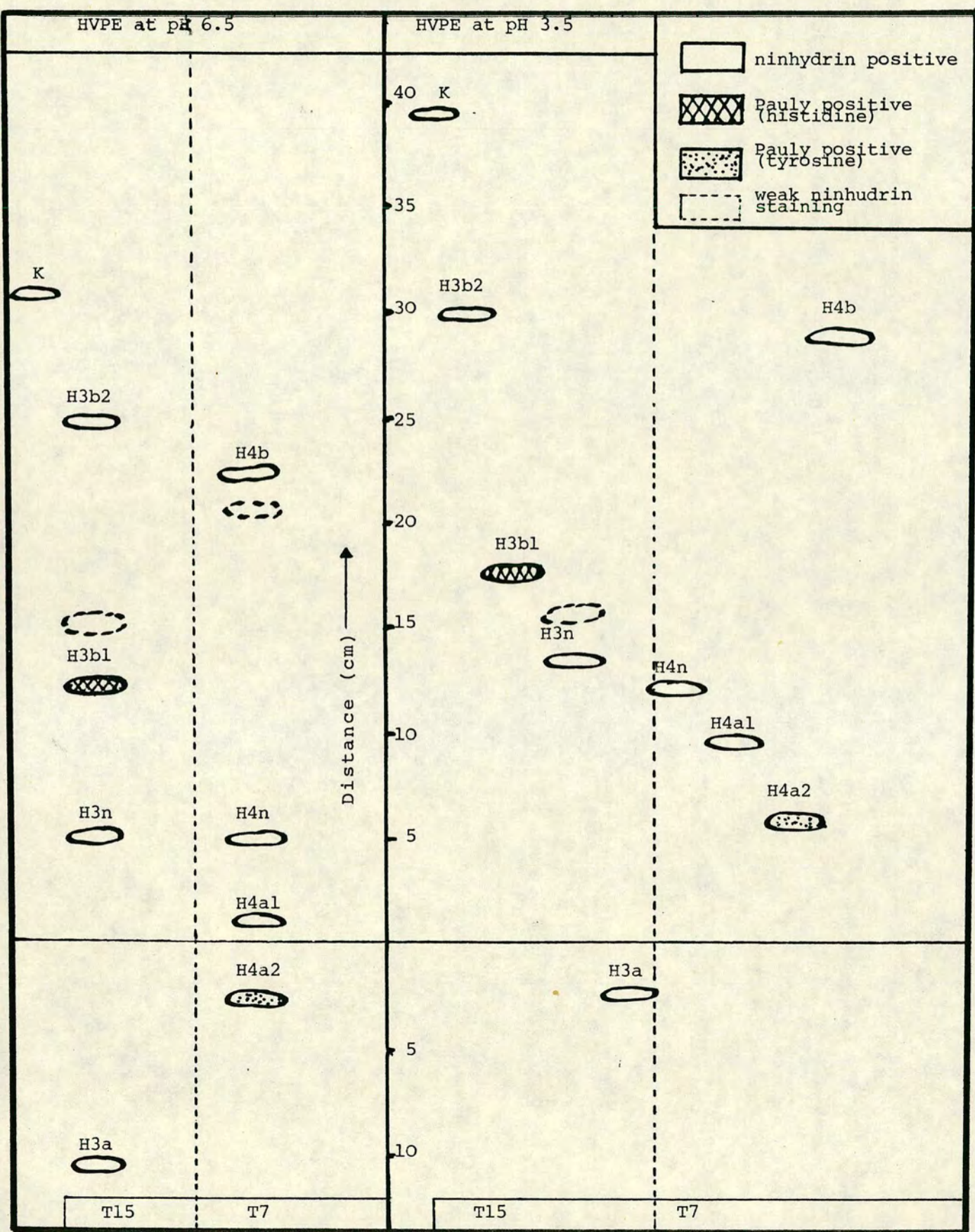


Fig. 3.10: Separation of peptides resulting from the thermolytic digestion of peptides of peak 3 (T15) and peak 4 (T7).

Table 3.7 : Electrophoretic mobilities, N-termini and qualitative amino acid analysis of the thermolytic peptides of tryptic peptides T15 and T7

tryptic peptide	thermolytic peptides	electrophoretic mobility		N- terminus	Qualitative amino acid analysis
		pH 6.5	pH 3.5		
T15	H3b1	0.28	0.46	Leu	H, A, L, S, P, T, E
	H3b2	0.75	0.74	Val	L, V
	H3n	0	0.33	Leu	L
	H3a	-0.62	0.05	Leu	A, F, E, D, C
T7	H4b	0.65	0.71	Thr	K, T
	H4a1	-0.15	0.23	Ile	A, I, T, E
	H4a2	-0.29	0.14	Phe	P, F, Y, D
	H4n	0	0.31	Val	V, T, P

Table 3.8 : Quantitative amino acid analysis of thermolytic peptides of tryptic peptides T15 and T5

Amino acid peptide	Asx	Thr	Ser	Glx	Pro	Gly	Ala	Val	Ile	Leu	Tyr	Phe	His	Lys	Arg
H3a	1.26			1.14			1.97			0.92		0.71			
H3b1		0.94	1.90	1.14	1.96		1.11			0.78		1.00			
H3b2								1.02						0.98	
H3n	(free leucine)														
H4a1		0.67		1.16			1.14		1.03						
H4a2	2.16				0.56						1.77	0.60			
H4n		1.41			0.99			1.60							

3.1.7.b(3) ADH-F' T7 thermolytic peptides

H4b: threonine was the N-terminus, and qualitative amino acid analysis revealed the presence of only lysine and threonine. In conjunction with the results derived from the rest of the peptides its sequence was deduced as: T-K.

H4n: sequential degradation of this peptide revealed the following sequence:

V-T-V-P
 $\overline{\text{V}} \quad \overline{\text{T}} \quad \overline{\text{V}} \quad \overline{\text{P}}$

This thermolytic peptide had not been previously isolated.

H4a1: the sequence was established as:

I-A-E-T
 $\overline{\text{I}} \quad \overline{\text{A}} \quad \overline{\text{E}} \quad \overline{\text{T}}$

As can be seen in Table 3.8 during quantitative amino acid analysis of this peptide a smaller amount of threonine than expected was recovered. It was thought possible that a peptide with I-A-E sequence, present in smaller amounts, contaminates the H4a1 yields.

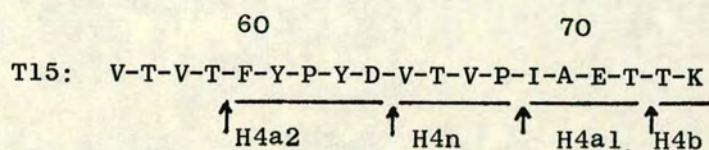
H4a2: in the quantitative amino acid analysis of this peptide abnormally high amounts of aspartic acid were recorded, but sequential degradation established the sequence as:

F-Y-P-Y-D
 $\overline{\text{F}} \quad \overline{\text{Y}} \quad \overline{\text{P}} \quad \overline{\text{Y}} \quad \overline{\text{D}}$

being therefore identical with peptide H13 purified by Thatcher (1980) after the thermolytic digestion of whole protein (ADH-S).

With /

With the help of the published sequence these peptides can be pieced together as follows:



As can be seen the N-terminal sequence is missing but the rest agrees well with the published sequence. It is worth noting that two thermolytic cleavage sites are after threonine residues (residues 59 and 62), resulting probably from a high enzyme substrate ratio during digestion.

3.1.7.c Thermolysin digestion of fraction pool 1 (mixed large tryptic peptides)

The remaining amount of fraction pool 1 (Fig. 3.3) containing the high molecular weight tryptic peptides of poor solubility was digested as a whole by thermolysin in the hope of isolating and sequencing useful fractions of peptides that were not purified by ion-exchange chromatography. As in section 3.1.7.a the reasons for using thermolysin were that thermolytic peptides were expected to cover most of the sequence and are easier to handle than those expected from chymotrypsin.

The enzymic digestion was performed in the urea buffer system in conditions described in section 2.3. After digestion the contents were freeze-dried, resuspended in 1 M acetic acid and were subsequently passed through a Sephadex G-25 SF column (diameter 15 mm, length 150 mm). /

(diameter 15 mm, length 150 mm). The contents of the fractions were electrophoresed on paper at pH 6.5. (Results can be seen in Fig. 3.11).

After collecting the fractions in a single pool (no advantage existed in creating separate pools since the majority of peptides are present in most fractions), the contents of the fractions were freeze-dried and redissolved in 1 ml of 1 M acetic acid.

Peptides were then purified with preparative HVPE at pH 6.5 and 3.5. Purity was assessed by N-terminus determination. Spots that were found to contain more than one peptide after HVPE at pH 3.5 were subsequently separated by BAWP chromatography on paper.

Qualitative and quantitative amino acid analysis determined the composition of the pure peptides (Tables 3.9, 3.10). These results and the coincidence of the N-termini and electrophoretic mobilities identified peptides HTHb5, HTHa4.1 with peptides H3b1 and H3a respectively, thermolytic fragments of tryptic peptide T15, isolated and sequenced as described in section 3.1.7.b(2).

Peptides HTHn4.1 (sequenced as $\overline{\text{V-A-E-K}}$), HTHa4.2 (sequenced as $\overline{\text{L-D}}$) and peptide HTHa4.3 (sequenced as $\overline{\text{L-D-V-E-P-Q}}$) are thermolytic fragments of the amounts of tryptic peptide T14b, present in pool 1, as can be seen by aligning them with the published sequence (Thatcher, 1980).

T14b: F-N-S-W-L-D-V-E-P-Q-V-A-E-K

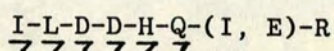
HTHa4.2

HTHa4.3 HTHn3.1

The /

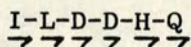
The recovery and sequencing of these peptides therefore completed the sequencing of peptide T14b. Only the first 7 residues were identified from the amount purified from pool 2, although amino acid composition did suggest then that no further substitution would be expected in this part of the sequence.

Peptide HTHa1 was sequenced as:



This peptide was digested further by thermolysin as it is evident from peptides:

HTHa2 sequenced as:

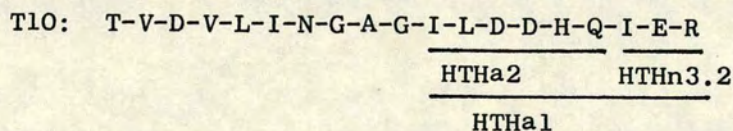


HTHn4.2 sequenced as:



(both are identical with peptides H22 and H23 described by Thatcher, 1980).

The sequence of these peptides agrees with the C-terminal part of tryptic peptide T10.



Part of the sequence of the T11a pseudotryptic peptide (T11b was identified and partially sequenced with peptides of pool 2) were /

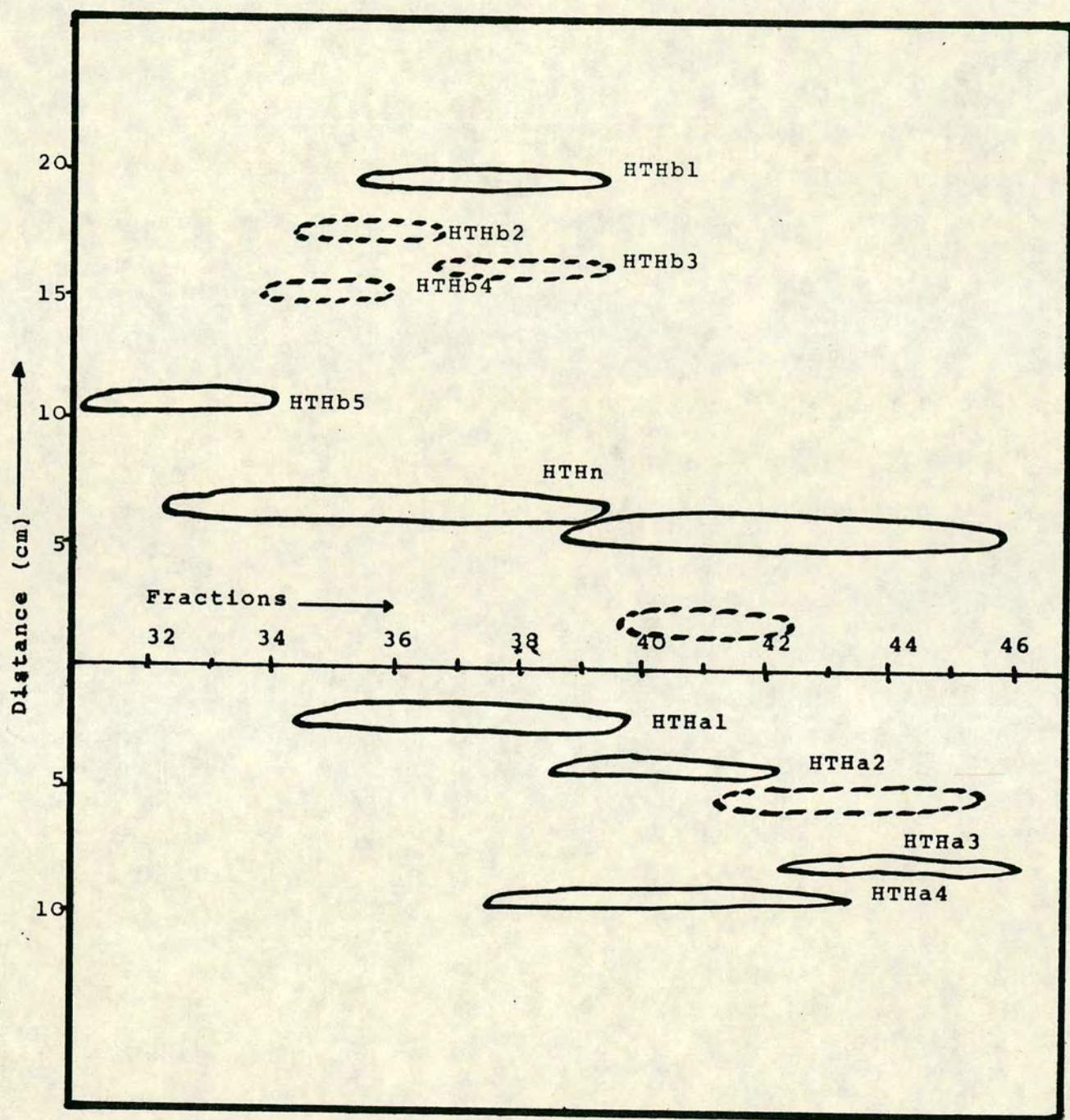


Fig. 3.11 ; HVPE at pH 6.5 of Sephadex G-25 SF gel filtration fractions of the thermolytic digest of fraction pool 1.

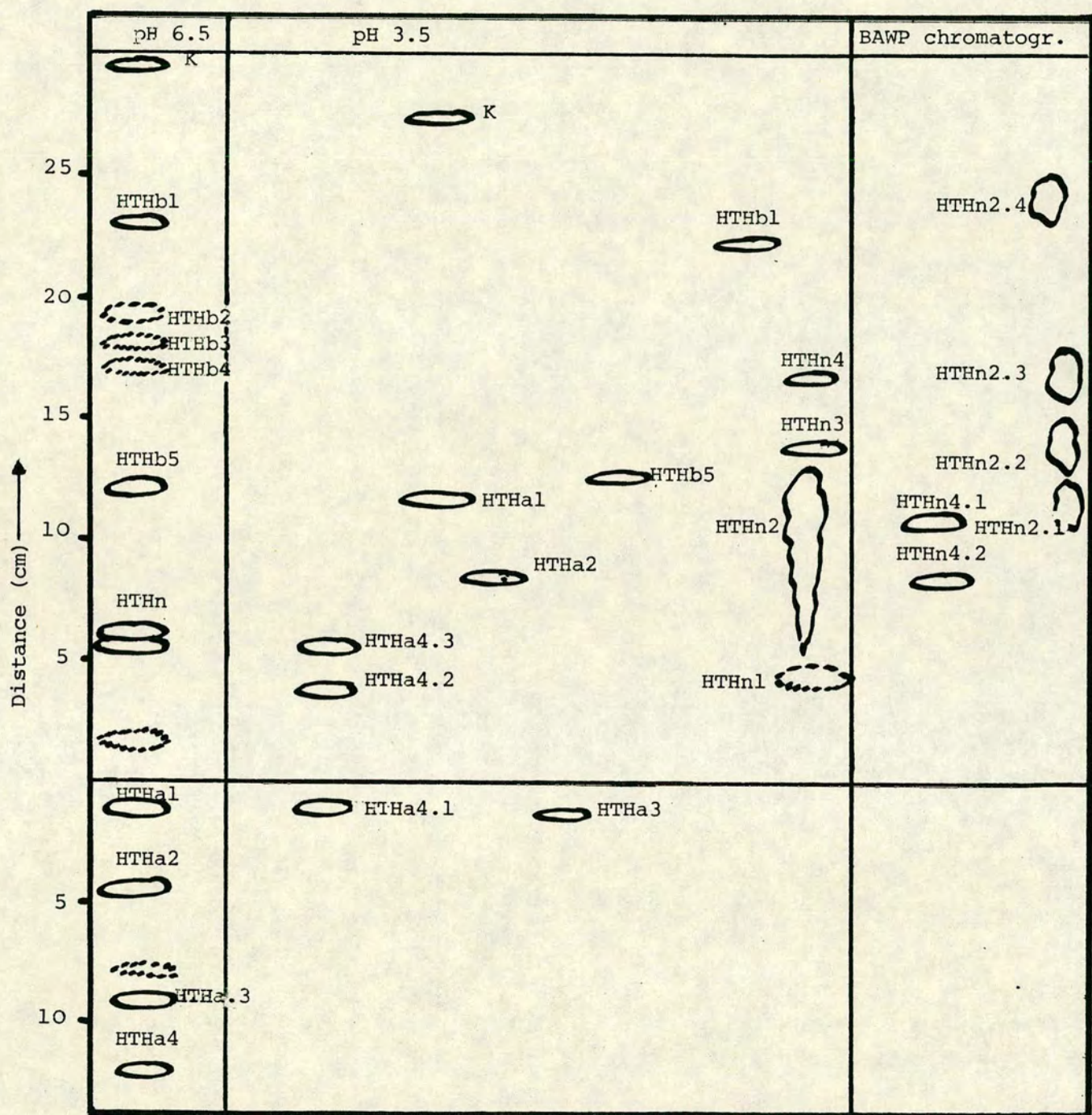


Fig. 3.12 : Separation of peptides present in the thermolytic digest of fraction pool 1.

Table 3.9 : Electrophoretic mobility, N-termini and Qualitative amino acid analysis of thermolytic peptides isolated from the digest of fraction pool 1.

Peptide	Electrophoretic mobility		N-terminus	Qualitative amino acid analysis
	pH 6.5	pH 3.5		
HTHb1	0.79	0.85	lost	lost
HTHb5	0.41	0.46	Leu	H, A, L, S(?), P, T, E
HTHn4.1	0	0.61	Val	K, A, V, E
HTHn4.2	0	0.61	Ile	R, I(?), E
HTHn2.3	0	0.31	Val	G, V, T
HTHa1	-0.24	0.44	Ile	R, H, L, I(?), E, D
HTHa2	-0.34	0.31	Ile	H, L, I(?), E, D
HTHa3	-0.51	-0.07	Ile	I, D, C
HTHa4.1	-0.62	-0.06	Leu	A, F, E, D, C
HTHa4.2	-0.62	-0.15	leu	L, D
HTHa4.3	-0.62	-0.22	Leu	L, V, P, E

(?) dubious identification

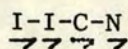
Table 3.10 : Quantitative amino acid analysis of the thermolytic peptides isolated from the digest of fraction pool 1

Amino acid peptide	Asx	Thr	Ser	Glx	Pro	Gly	Ala	Val	Ile	Leu	Tyr	Phe	His	Lys	Arg
HTHb5		0.74	0.66	0.98	1.82		1.05			1.70			1.43		
HTHn4.1				0.98			1.11	0.91						0.94	
HTHn4.2				0.97					1.03						1.35
HTHn2.3		1.29				0.94		1.06							
HTHa1	2.20			1.86					1.68	0.92			1.02		2.15*
HTHa2	2.10			0.93					1.11	0.87			0.99		
HTHa3	1.17								1.82						
HTHa4.1	1.32			0.99			1.81			0.92		0.95			
HTHa4.2	1.20									0.80					
HTHa4.3	1.27			1.89	0.90			0.97		0.93					

* Arginine amounts were overestimated because of an unusually high overlapping NH_3 peak

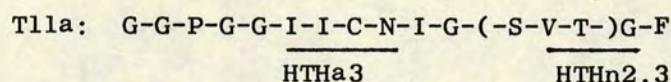
were provided with the isolation of peptides HTHa3 and HTHn2.3.

Peptide HTHa3 was sequenced as:



(cysteine was identified through qualitative amino acid analysis).

HTHn2.3 sequenced as V-T-G, provided positive sequence information for the only uncertain area of the ADH primary structure as determined by protein chemistry techniques (Thatcher, 1980).



A number of positively charged thermolytic fragments present in the digest were not identified because of the small amounts recovered. The neutral band of peptides resolved by HVPE at pH 3.5 and BAWP chromatography contained as expected free neutral amino acids (glycine, valine) partially digested fragments of tryptic peptides (HTHn) and small amounts of dipeptides (HTHn2.1: T-G HTHn2.2: V-T) which position in the sequence is ambiguous.

3.1.8 Summary of sequence information

The experiments previously described made possible the recovery of sequence information of about 80% of the molecule of ADH-F'. In this part of the sequence the only deviation from the published ADH-S primary structure (Thatcher, 1980; Benyajati et al, 1981) was the substitution of alanine for glutamic acid in position 51. /

Acetyl-S-F-T-L-T-N-K-N-V-I-F-V-A-G-L-G-G-I-G-L-D-T-S-K-E-L-L-K-R-D-L-K-N-I-V-
 ↓
-I-L-D-R-I-E-N-P-A-A-I-A-E-L-K-E-I-N-P-K-V-T-V-T-F-Y-P-Y-T-V-T-V-P-I-A-E-T-T-K-
-L-L-K-T-I-F-A-Q-L-K-T-V-D-V-L-I-N-G-A-G-I-L-D-D-H-Q-I-E-R-T-I-A-V-N-Y-T-G-I-V-
-N-T-T-T-A-I-I-D-F-W-D-K-R-K-G-G-P-G-G-I-I-C-N-I-G-(S, V, T,)-G-F-N-A-I-Y-Q-V-
-P-V-Y-S-G-T-K-A-A-V-V-N-F-T-S-S-L-A-K-L-A-P-I-T-G-V-T-A-Y-T-V-N-P-G-T-T-R-T-T-
-L-V-H-K-F-N-S-W-L-D-V-E-P-Q-V-A-E-K-L-L-A-H-P-T-Q-P-S-L-A-C-A-E-N-F-V-K-A-I-E-
-L-N-Q-N-G-A-I-W-K-L-D-L-G-T-L-E-A-I-Q-W-T-K-H-W-D-S-G-I-COOH

Fig. 3.13 : Extent of sequence information on the ADH-F' enzyme.

- ..G-V.. sequence suggested by amino acid analysis and Edman degradation
 of tryptic peptides
-N-I...- HVPE and Chromatography suggest identical sequence with the
 corresponding ADH-S tryptic peptide
-H-W...- HVPE and Chromatography as well as qualitative amino acid ana-
 :lysis suggest sequence identical with the corresponding ADH-S
 tryptic peptide
 Non-underlined sections show DADH amino acid sequence (Thatcher,
 1980) for which definitive information is lacking in ADH-F'.

51. The extent of the sequence information, the amino acid analysis and the fact that the discovered substitution fully explains the electrophoretic mobility of ADH-F', tend to lead to the conclusion that no further substitutions exist in the F' molecule (kinetic behaviour and thermal denaturation experiments, discussed in Chapter 4 support this conclusion). Figure 3.13 summarises the sequence information obtained from this series of experiments.

3.2 Structural studies on the ADH-US enzyme

ADH-US (ultra-slow) is a rare variant in the ADH locus of *Drosophila melanogaster* detected by David et al (1978) in flies of the Colmar (France) population. The ADH-US enzyme can be easily distinguished in gel electrophoretograms as it is more positively charged than ADH-S.

Chambers et al (1981) surveying several kinetic properties of known ADH variants in *Drosophila melanogaster*, reported that the enzymic behaviour of the ADH-US enzyme was the only one to fall outside the two main functional groups (F-like and S-like) in which the studied variants of ADH seemed to belong. The properties surveyed were the specific activity and the substrate preference of these enzymes.

There are three kinds of single substitutions on the two main sequences (F and S) that can lead to the electrophoretic behaviour exhibited by the US variant.

From ADH-S /

From ADH-S negative → neutral
 neutral → positive

From ADH-F negative → positive (although such substitutions are
 very rare).

Unfortunately, it has not been possible to isolate amounts of pure ADH-US adequate for major sequencing studies of a considerable part of the sequence in the manner reported for ADH-F' in section 3.a. This variant did not respond to the purification techniques outlined in Chapter 2 and in each attempt considerable losses were recorded in ammonium sulphate precipitation steps although the pH was adjusted in this case. The slow filtration usually ended with no detectable ADH activity being eluted. Repeated attempts, even with large batches (1 kg of larvae), gave disappointing results. It became therefore evident that the US variant was producing an ADH enzyme much less stable than the ones investigated so far.

Affinity methods for the purification of ADH described by Leigh-Brown and Lee (1979) and successfully applied to the purification of ADH-F, ADH-S and ADH-F' (Chapter 4) failed to give results because the enzyme seemed to fall quickly to the less active forms (ADH-3 and 1). These forms were not retained or retarded by the ligand.

Small amounts (6 mg) were purified from a starting material of 100 g of *Drosophila melanogaster* 3rd instar larvae by the following scheme: after protamine sulphate precipitation of nucleic acids the ammonium sulphate precipitation steps were performed as usual. The desalted precipitate of the 60% ammonium sulphate saturation step was /

was passed through a DEAE-52 (length 10 cm; diameter 1.5 cm) that did not retain the enzyme but successfully removed a significant part of the soluble protein. The effluent was then freeze-dried, oxidised with performic acid and resuspended in 1 M acetic acid. The sample was then passed through a Sephadex G-100 coarse column (length 110 cm; diameter 2.5 cm). Fractions containing the denatured ADH were identified through SDS electrophoresis and were pooled and freeze-dried.

The small amount thus recovered was digested with trypsin and the digest was separated on a Sephadex G-25 SF column. High voltage paper electrophoresis of the fractions at pH 6.5 is shown in Fig. 3.14. The region of the electrophoretogram containing the basic peptides resembles the one present in similar electrophoretograms of tryptic digests of ADH-S (Fig. 3.4). From this region the "diagnostic" S peptide T14a was recovered, identified and analysed (Table 12) along with T8, T6 and T2c. The "pseudotryptic" peptide T12b (not detected in ADH-F' tryptic digests) was found to possess the same electrophoretic mobility at pH 3.5 and 6.5 as peptide T2c. The two peptides were finally separated by paper chromatography. Peptides T9 and T13b were identified by their electrophoretic mobility and N-termini but there were insufficient amounts for amino acid analysis. Peptides T12 and T13b were not recovered. Among the electrophoretically neutral peptides T2b and T3 were recovered, identified and analysed. T16 and T13a were recovered also but in small quantities and analysis gave poor results. Among the acidic peptides usually present in tryptic digestions of ADH-S (T14b, T5, T17) /

T17) T14b and T5 were recovered in low yields in this experiment. They were identified by their electrophoretic mobilities and N-termini. Amino acid analysis was unsatisfactory since, as with T16 and T13a, amino acid release during a 24-hour acid hydrolysis had been adversely affected by the small amounts present. A new acidic spot (Tx) was shown to be a partially digested part of the enzyme.

These results show that the substitution responsible for the electrophoretic mobility of ADH-US must be sought in the part of the sequence covered by the tryptic peptides that display poor solubility under the conditions used in this experiment. The small amounts of starting material in conjunction with the means available made the recovery of such peptides in usable amounts impossible.

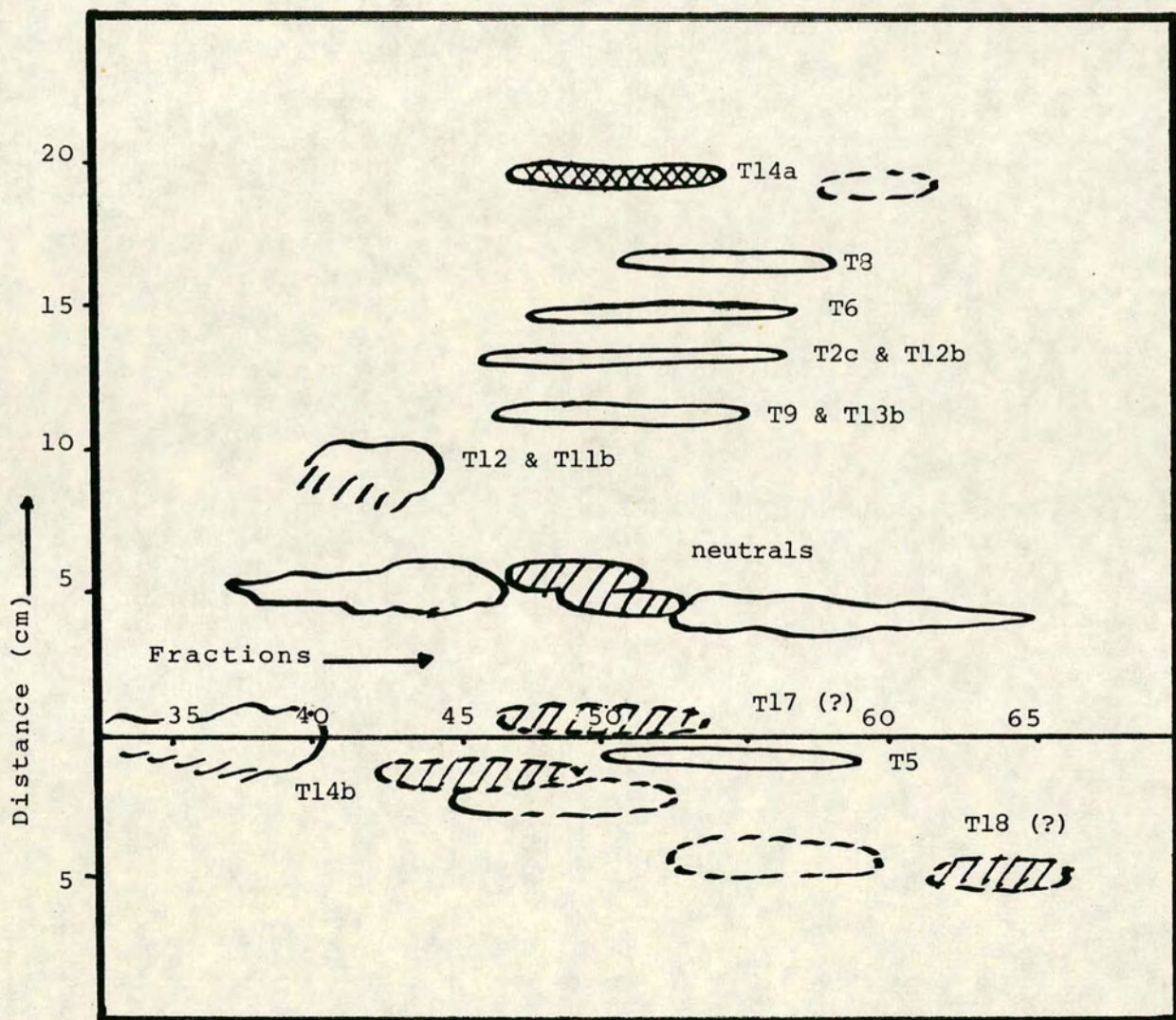


Fig 3.14 : HVPE at pH 6.5 of the tryptic digest of oxidized Drosophila ADH-US after fractionization by Sephadex G-25 SF gel filtration.

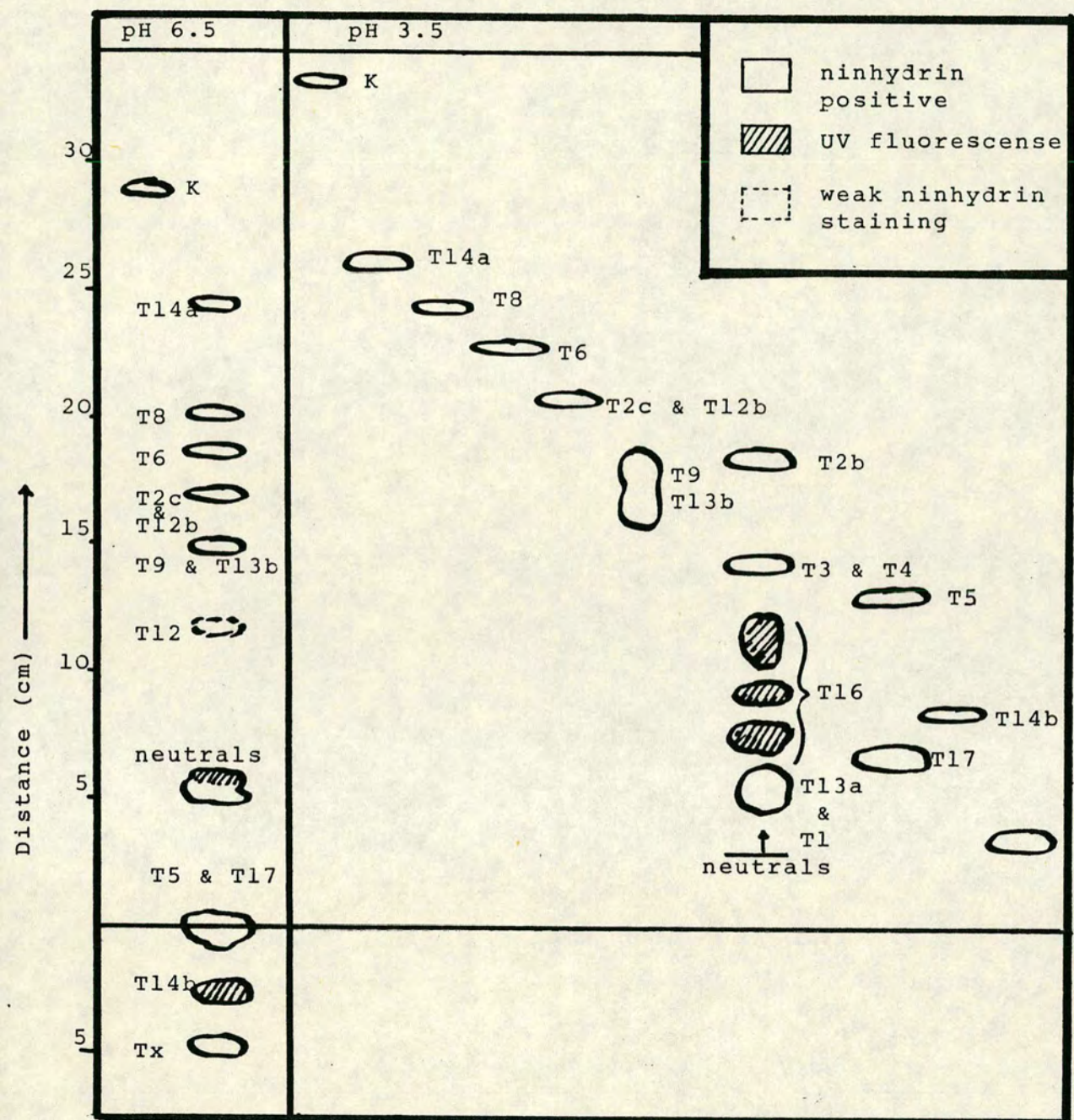


Fig. 3.15 : HVPE at pH 6.5 and 3.5 of soluble tryptic peptides of oxidized ADH-US.

Table 3.11 : Quantitative amino acid analysis of recovered soluble tryptic peptides of oxidized ADH-US

peptide	Amino acid		Asx	Thr	Ser	Glx	Pro	Gly	Ala	Val	Ile	Leu	Tyr	Phe	His	Lys	Arg
	(a)	(b)															
T14a	(a)	(b)		1.59						0.73		1.54			1.01	0.71	
				2						1		1			1	1	
T8	(a)	(b)										1.57				1.42	
												2				1	
T6	(a)	(b)	0.95				1.23		0.93		0.90					0.88	
			1				1		1		1					1	
T2c	(a)	(b)				1.10						1.94				0.92	1.03
						1						2				1	1
T2b	(a)	(b)				1.33						1.59				1.08	
						1						2				1	
T3	(a)	(b)	1.16									1.01				0.82	
			1									1				1	
T14b	(a)	(b)	1.83		1.20	1.71	0.99		1.84	1.19		1.18		1.29		1.18	
			2		1	3	1		1	2		1		1		1	
T5	(a)	(b)	1.53			2.20	1.16		2.10		1.62	0.81				1.18	
			1			2	1		3		2	1				1	
T12b	(a)	(b)		1.08	1.55				1.09			1.04				0.86	
				1	2				1			1				1	

(a): residues / mole peptide calculated from analysis results.

(b): composition deduced from sequence information.

CHAPTER 4: Kinetic and conformational studies on ADH alleloenzymes
(ADH-F; ADH-S; ADH-F')

4.1 Separation of ADH-5

The preliminary kinetic investigation of the ADH-F, ADH-S and ADH-F' involved the determination of pH effects on the apparent Michaelis constants and maximal rates of these enzymes in relation to isopropanol, as well as their kinetic behaviour in relation to NAD^+ (the in vivo cofactor) and two NAD^+ -analogs. Only ADH-5 containing preparations of the alleloenzymes were used. According to the current theory of isoenzyme formation in *Drosophila* ADH (Chapter 1), ADH-5 is regarded as the product of the gene expression, with the ADH-3 and ADH-1 forms being derived through post-translational modification.

ADH-5 was isolated from purified enzyme preparations containing all three forms as described in section 2.4. It was retained by the ligand while ADH-1 and ADH-3 were slightly retarded. Fig. 4.1 shows a separation of ADH-F'-5, which is typical.

The UV spectra of ADH-1 (the inactive fraction) and ADH-5 (after dialysis that removed NAD and pyrazole) were compared. The inactive enzyme in the most anodal form shows a decrease in absorption at 280 nm (decreased A_{280}/A_{240} ratio), while the 290-286 shoulder due to the tryptophan side chain absorption has totally disappeared. /

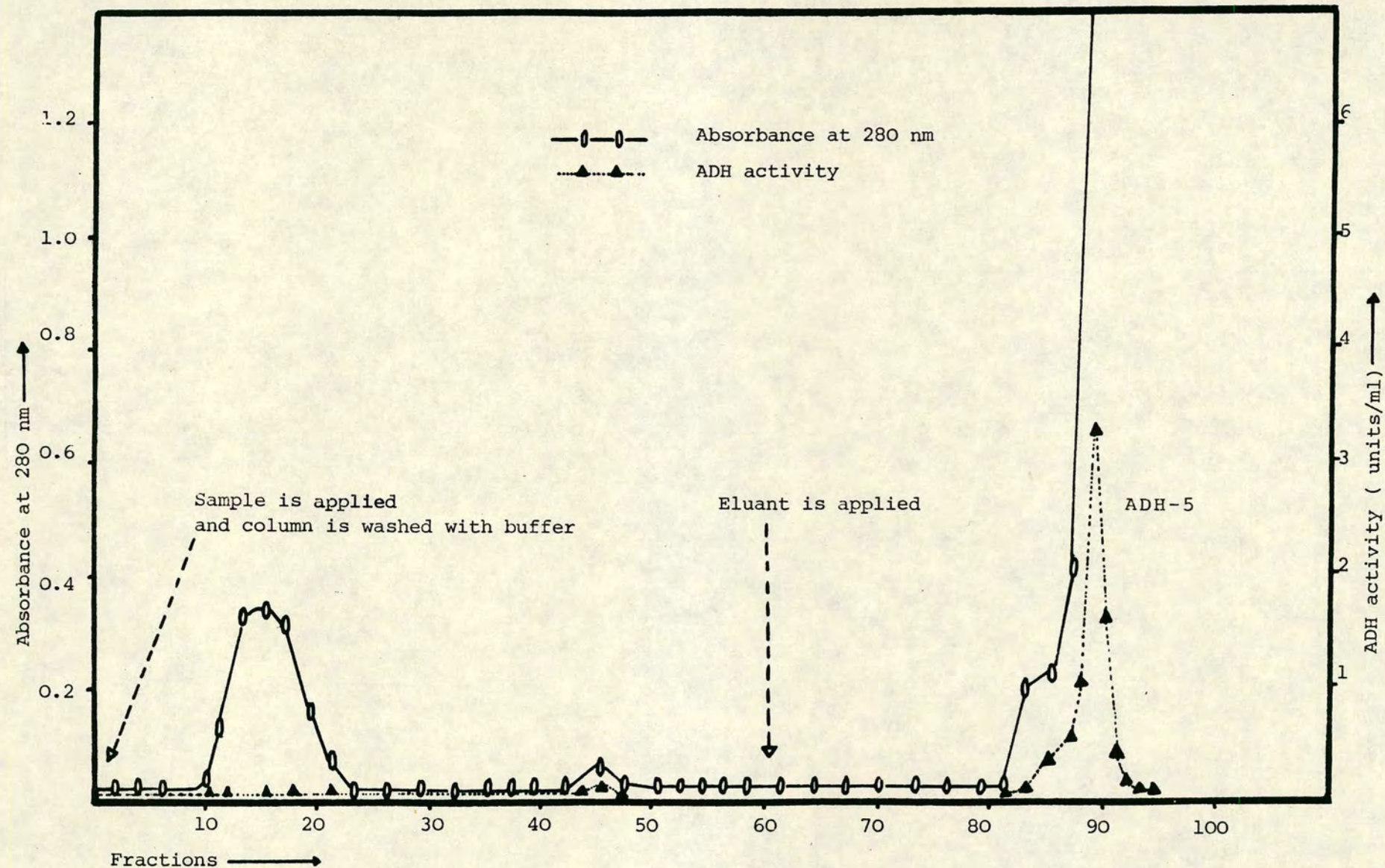


Fig. 4.1 : Separation of ADH-5-F' by 8-hexyl-6-(amino)-AMP Sepharose 4B column from the other molecular forms of Drosophila ADH-F'.

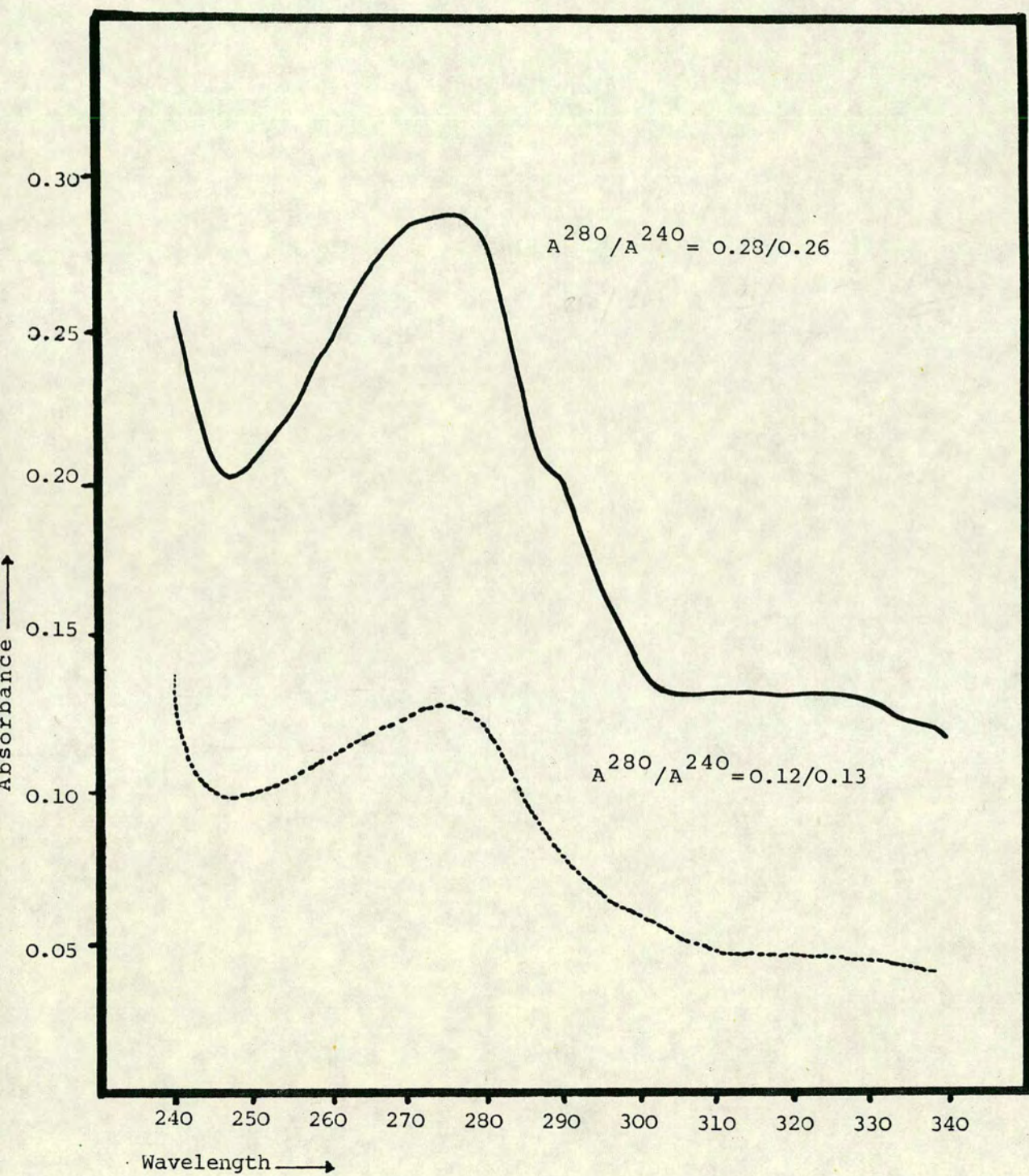


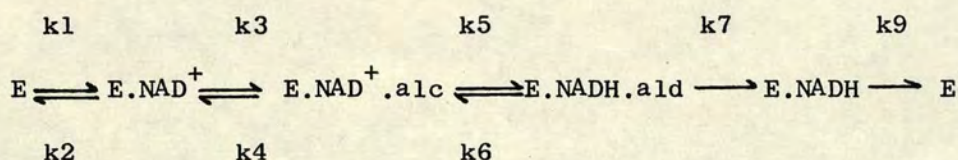
Fig. 4.2 : UV-spectrum of ADH-5-F' purified by affinity chromatography (solid line) and of inactive ADH-F' (dotted line) not retained by the ligand.

disappeared. But it must be noted that the purified ADH-F' preparation used for the isolation of ADH-5 had been previously freeze-dried, and the resulting ADH forms may not correspond with the in vivo ones.

4.2 pH effects on the ADH kinetic constants for isopropanol

The Michaelis constant (K_m) is usually defined operationally as the substrate concentration that gives half the maximal rate under defined experimental conditions. In the simple one-substrate Michaelis-Menten mechanism, K_m equals K_s the dissociation constant of the substrate-enzyme binary complex (not though in the Briggs-Haldane treatment of this mechanism).

But in two-substrate enzymic reactions (like the one that ADH catalyses) K_m has invariably a more complex kinetic meaning.



(such an illustration incorporates the assumption that initial rates were measured before products build up sufficiently to start the reversing steps of the mechanism).

Scheme 1: reaction mechanism prevailing in NAD^+ -dependent dehydrogenases (alc = alcohol, ald = aldehyde)

But these more complicated reactions can be kinetically described in the framework of the simple Michaelis-Menten mechanism, provided that one of the substrates is present in saturating concentrations.

In /

In such cases, K_m and k_{cat} are apparent, not real, constants. In the compulsory-ordered-ternary complex mechanism illustrated on page 106 in Scheme 1, the apparent Michaelis constant of the enzyme for alcohol substrates ($K_m^{alc}(\text{app})$) in saturating concentrations of NAD^+ and a steady state for E, is defined as:

$$K_m^{alc}(\text{app}) = \phi_{alc} / \phi_o$$

$$\text{where } \phi_{alc} = k_5k_7 + k_5k_9 + k_6k_9 + k_7k_9 / k_5k_7k_9$$

$$\phi_o = k_4k_6 + k_9k_7 + k_5k_7 / k_3k_5k_7 \quad (\text{Dalziel, 1957}).$$

This ordered mechanism is followed by the liver alcohol, lactate and malate dehydrogenases. The coenzyme (NAD^+) by binding first induces conformational changes that allow the binding of the second substrate (Adams et al, 1973; White et al, 1976). Detailed studies showed that some differences do exist among these enzymes regarding the dissociation rates of the ternary complexes (Holbrook and Gutfreund, 1973; Whitaker et al, 1974).

Such a mechanism is suggested in *Drosophila* ADH from the relative independence of k_{cat} for alcohol oxidation (maximum rate of the forward reaction) from pH alterations, especially above pH 5.5 (discussed later in this chapter). This behaviour is consistent with an LDH or an LADH mechanism where the "slow" NADH release is the rate determining step (Holbrook and Gutfreund, 1973). Similar k_{cat} independence from pH has been observed by Schwert et al (1967) for beef H4 LDH. The observed substrate activation (this chapter and in Appendix 3) in saturating concentrations of both substrates is /

is again consistent with this mechanism (Dalziel and Dickinson, 1966).

The maximum rate of the forward reaction (alcohol to aldehyde), represented as V_f or k_{cat} , gives the maximum number of substrate molecules converted to products per active site per unit time. Therefore, both K_m ($\frac{S(x)}{app}$) and k_{cat} can give some idea about the capabilities of the enzyme towards that substrate x in vitro. In vivo these estimates may never be achieved but they can be treated as upper limits.

K_m^{alc} and k_{cat} for the aforementioned alleloenzymes were determined in part of the pH range (4.85-7.45 for ADH-S; 4.85-6.9 for ADH-F; 6.14-6.9 for ADH-F') with isopropanol as the alcohol substrate. The effects of the pH on the kinetic properties of the enzyme can be due to the changes in the ionisation state either of the free enzyme, the enzyme-substrate complex or of the substrate, although substrate ionisation does not occur in this case and can be discounted as a cause. Usually enzymes remain active in the area of the pH range, where the ionisation state of groups closely related, either functionally or structurally, with the active site has no detrimental effect on the substrate affinity and reaction rates.

The ionisation state of the free enzyme can be followed by the k_{cat}/K_m ($\frac{app}{app}$) ratio, a second order rate constant that relates reaction rates to the free rather than the total enzyme concentration. In the study of pH dependencies springing from the simple Michaelis-Menten mechanism (Scheme 2) /

Table 4.1 : K_m (app), k_{cat} , k_{cat}/K_m (app) values for isopropanol as the alcohol substrate in saturating concentrations of NAD⁺ for ADH-F, S, and F'

(A) K_m (app) (in mM)

pH	ADH-F	ADH-S	ADH-F'
4.85	296.8 + 96.4	399.3 + 78.2	-
5.35	425.0 + 51.3	270.3 + 42.5	-
6.14	61.1 + 6.3	22.7 + 2.5	29.8 + 4.6
6.55	13.7 + 1.3	11.9 + 1.8	12.2 + 2.3
6.90	12.6 + 0.2	5.8 + 0.6	9.7 + 1.3
7.45	-	2.2 + 0.3	-

(B) k_{cat} (in sec^{-1})

pH	ADH-F	ADH-S	ADH-F'
4.85	1.3 + 0.2	1.2 + 0.1	-
5.35	4.7 + 0.5	2.4 + 0.15	-
6.14	9.8 + 0.4	2.7 + 0.1	3.2 + 0.2
6.55	9.5 + 0.4	3.6 + 0.15	4.0 + 0.1
6.90	9.7 + 0.1	4.1 + 0.1	4.2 + 0.2
7.45	-	3.9 + 0.1	-

(C) k_{cat}/K_m (app) (in $\text{sec}^{-1} \cdot \text{M}^{-1}$) (of mean values)

pH	ADH-F	ADH-S	ADH-F'
4.85	4.3	2.5	-
5.35	11.0	8.9	-
6.14	160.4	118.9	117.4
6.55	693.4	302.5	327.9
6.90	769.8	706.9	433.0
7.45	-	1.772.7	-

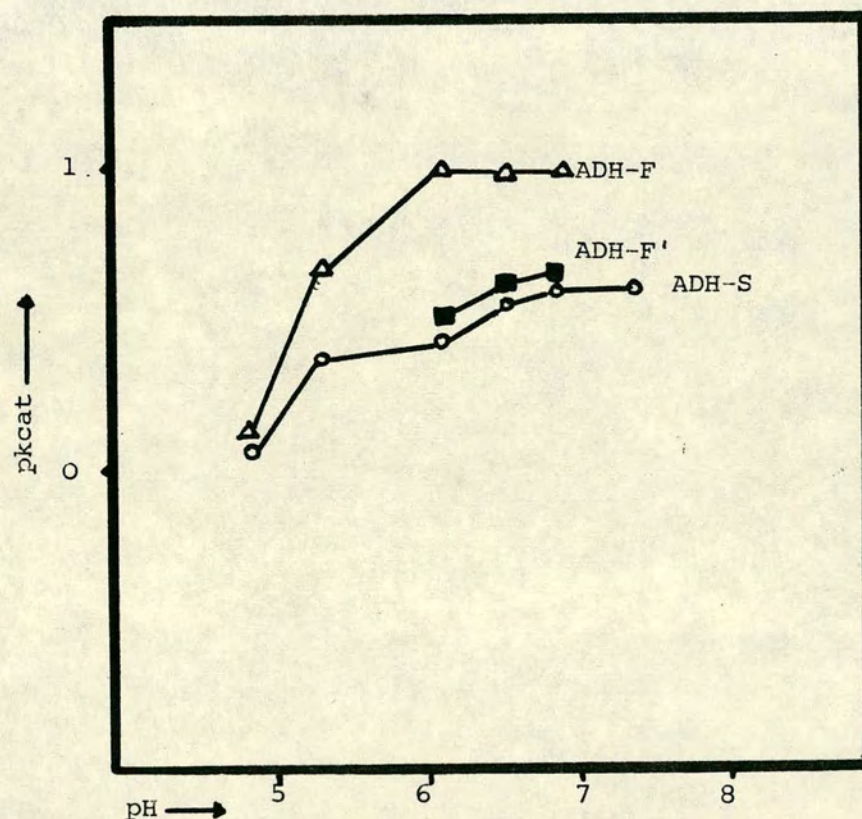
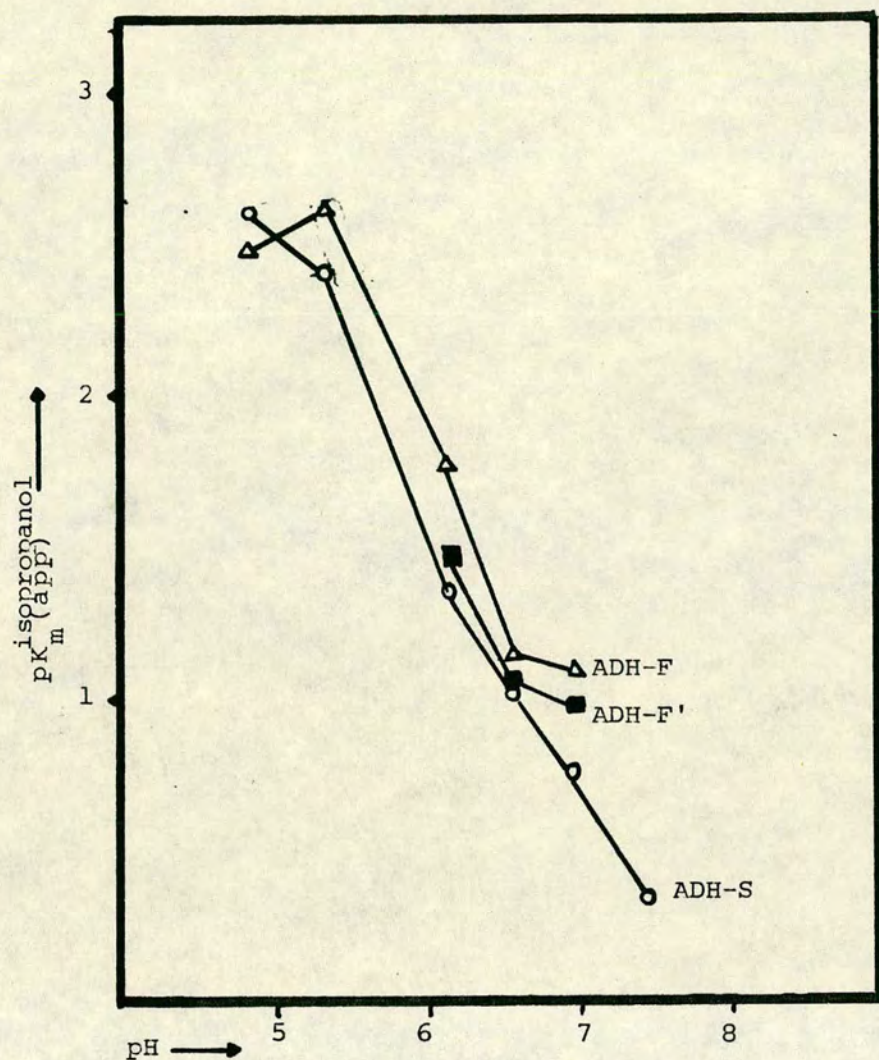
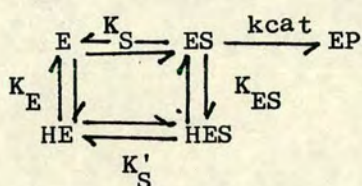


Fig. 4.3 : (a) $pK_m^{\text{isopropanol}}$ plotted against pH.
 (b) pK_{cat} plotted against pH.

simple Michaelis-Menten mechanism (Scheme 2)



$K'_S K_S$ = enzyme-substrate complex dissociation constant

K_E = free enzyme ionisation constant

K_{ES} = enzyme substrate complex ionisation constant

Scheme 2

this constant is defined as:

$$(k_{\text{cat}}/K_m)_H = (k_{\text{cat}}/k_s) K_E / K_E + H^+ \quad (\text{Dixon, 1953}).$$

The obvious assumption in such a mechanism is that there is only one ionic form of the enzyme active.

Results for k_{cat} , K_m (app) and k_{cat}/K_m (app) are summarised in Table 4.1. Figure 4.3 shows $p k_{\text{cat}}$ and $p K_m$ (app) plotted against pH. These results showed a decrease in affinity for isopropanol for both ADH-S and ADH-F below pH 6.5. This increase in K_m (app) values in this part of the pH range is consistent with an ionisable group of pK_a around 6.0. K_m (app) values seem to stabilise after pH 6.5 for ADH-F, but they continue falling for ADH-S. ADH-F', although not investigated to the same extent as the major alleloenzymes, shows a behaviour similar to that of the S variant.

k_{cat} values remain stable for both alleloenzymes above pH 5.5. Below pH 5.5 there is a drop, more pronounced in the case of ADH-F, but it results from some enzyme instability. This instability may be caused by random dissociation and association of the subunits (monomers have low activity) observed in low pHs (4) by Thatcher and Sheikh (1981). Therefore values for K_m (app) in this region might have been influenced by this factor.

Figure /

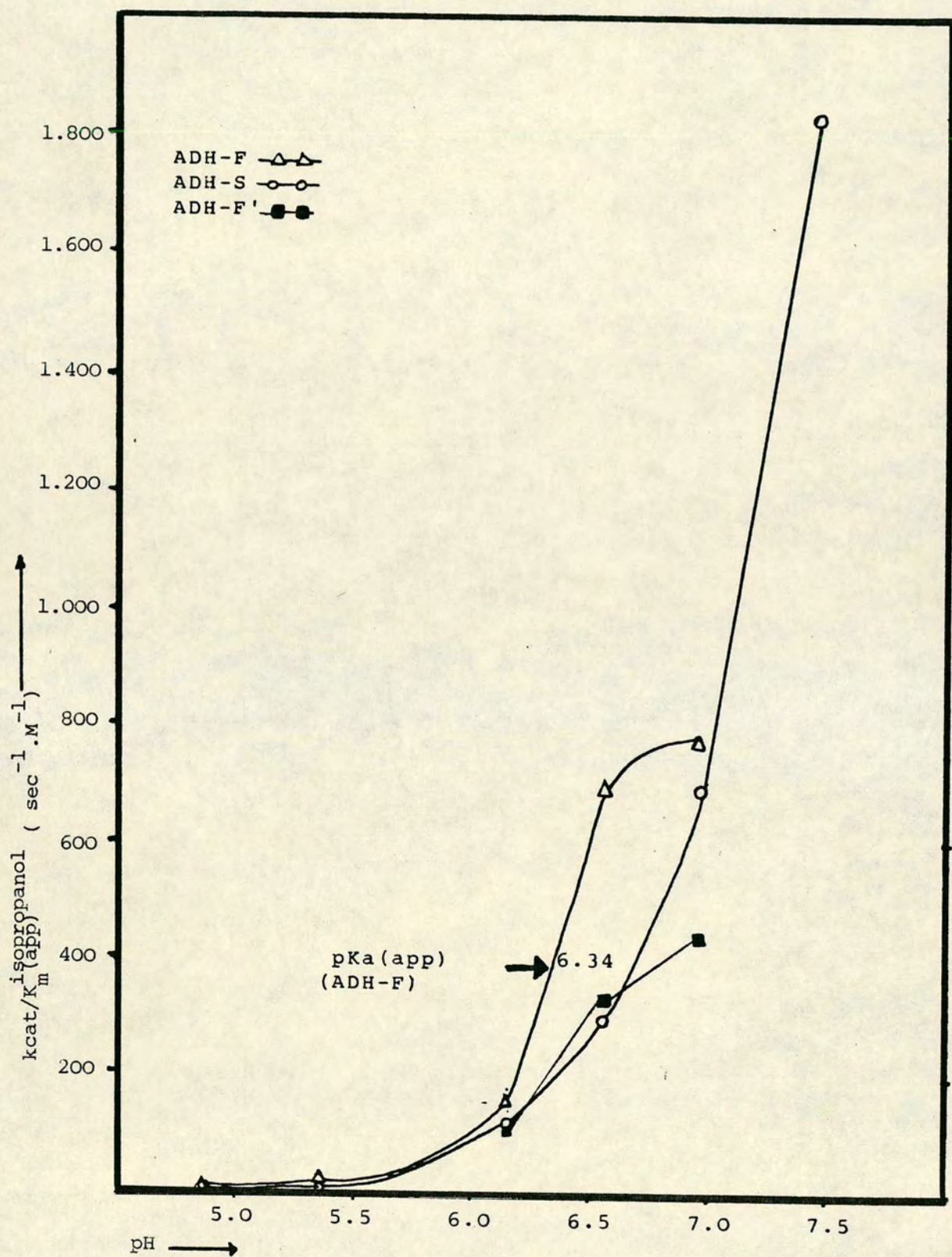


Fig. 4.4 : $k_{cat}/K_m^{isopropanol}$ for ADH-S, F and F' plotted against pH

Figure 4.4 shows k_{cat}/K_m (app) ^{isopropanol} values plotted against pH. It provides a rough estimate of the pK_a (app) of the free enzyme for ADH-F (around 6.3). The pK_a (app) of the ADH-S enzyme cannot be calculated from the available data, although it must be assumed that it lies in the basic sector of the pH range. The ionisation of ADH-F' resembles that of ADH-S.

Measurements and results calculated as mentioned in Chapter 2 when shown as reciprocal Lineweaver-Burke plots (Appendix 2) reveal that substrate activation occurs when both substrates are present in saturating concentrations. Such activation, if introduced in the calculation of the kinetic constants, tends to overestimate K_m and k_{cat} . The substrate range employed in these studies made it possible to obtain results not influenced by this phenomenon and was suggested by preliminary investigation.

4.3 Determination of k_{cat} , K_m (app) and k_{cat}/K_m of the alleloenzymes for NAD^+ and NAD^+ -analogs

These experiments were carried out in order to detect possible differences among the ADH variants in the binding of the nicotinamide moiety of the NAD^+ molecule. The ratio of activity between analogs with different substitutions in position 3 was used to investigate similarities among lactate dehydrogenases by Kaplan and Ciotti (1961).

The NAD^+ -analogs used here have also alternative groups replacing /

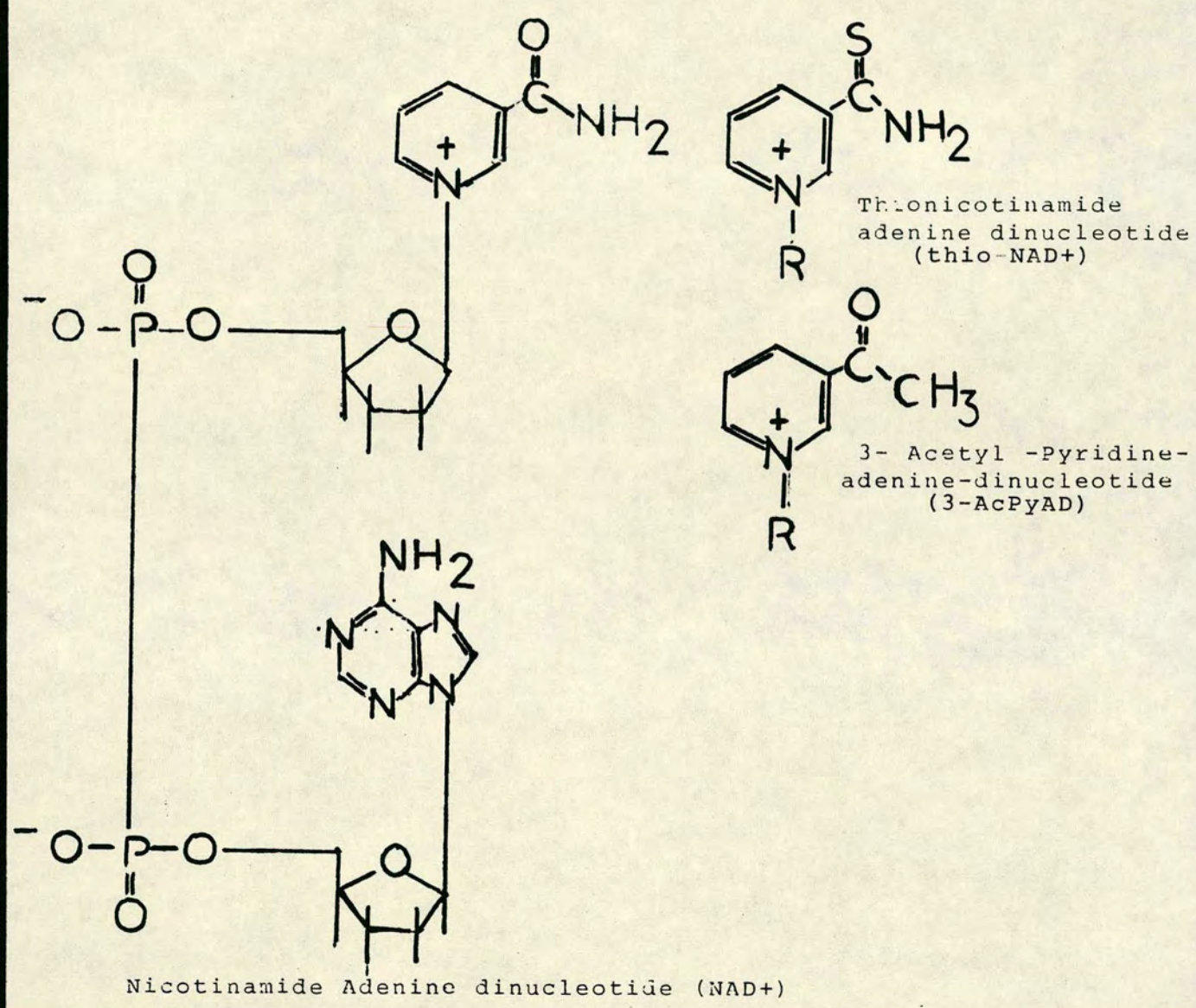


Fig 4.5.a . : Chemical formulas of NAD⁺ and the NAD⁺ analogs used .

Table 4.2.(a) : $K_m(\text{app})$, k_{cat} , $k_{\text{cat}}/K_m(\text{app})$ for NAD^+
at pH 7.45 (ionic strength 0.1)

	ADH-F	ADH-S	ADH-F'
$K_m(\text{app})$ (in mM)	0.118 ± 0.013	0.094 ± 0.016	0.091 ± 0.008
k_{cat} (sec^{-1})	10.42 ± 0.58	3.72 ± 0.46	4.10 ± 0.16
$k_{\text{cat}}/K_m(\text{app})$ ($\text{sec}^{-1} \cdot \text{M}^{-1}$)	88,305.1	39,574.5	45,054.9

Table 4.2.(b) : $K_m(\text{app})$, k_{cat} , $k_{\text{cat}}/K_m(\text{app})$ for thio- NAD^+
at pH 7.45 (ionic strength 0.1)

	ADH-F	ADH-S	ADH-F'
$K_m(\text{app})$ (in mM)	0.093 ± 0.007	0.081 ± 0.025	0.072 ± 0.009
k_{cat} (sec^{-1})	9.68 ± 0.29	10.15 ± 1.74	11.0 ± 0.64
$k_{\text{cat}}/K_m(\text{app})$ ($\text{sec}^{-1} \cdot \text{M}^{-1}$)	104,086.0	125,808.6	152,777.8

Table 4.2.(c) : $K_m(\text{app})$ and k_{cat} for 3-acetyl pyridine
dinucleotide at pH 7.45 (ionic strength 0.1)

	ADH-F	ADH-S	ADH-F'
$K_m(\text{app})$ (in mM)	0.886 ± 0.210	0.321 ± 0.057	0.676 ± 0.151
k_{cat} (sec^{-1})	1.66 ± 0.16	1.56 ± 0.08	1.73 ± 0.13

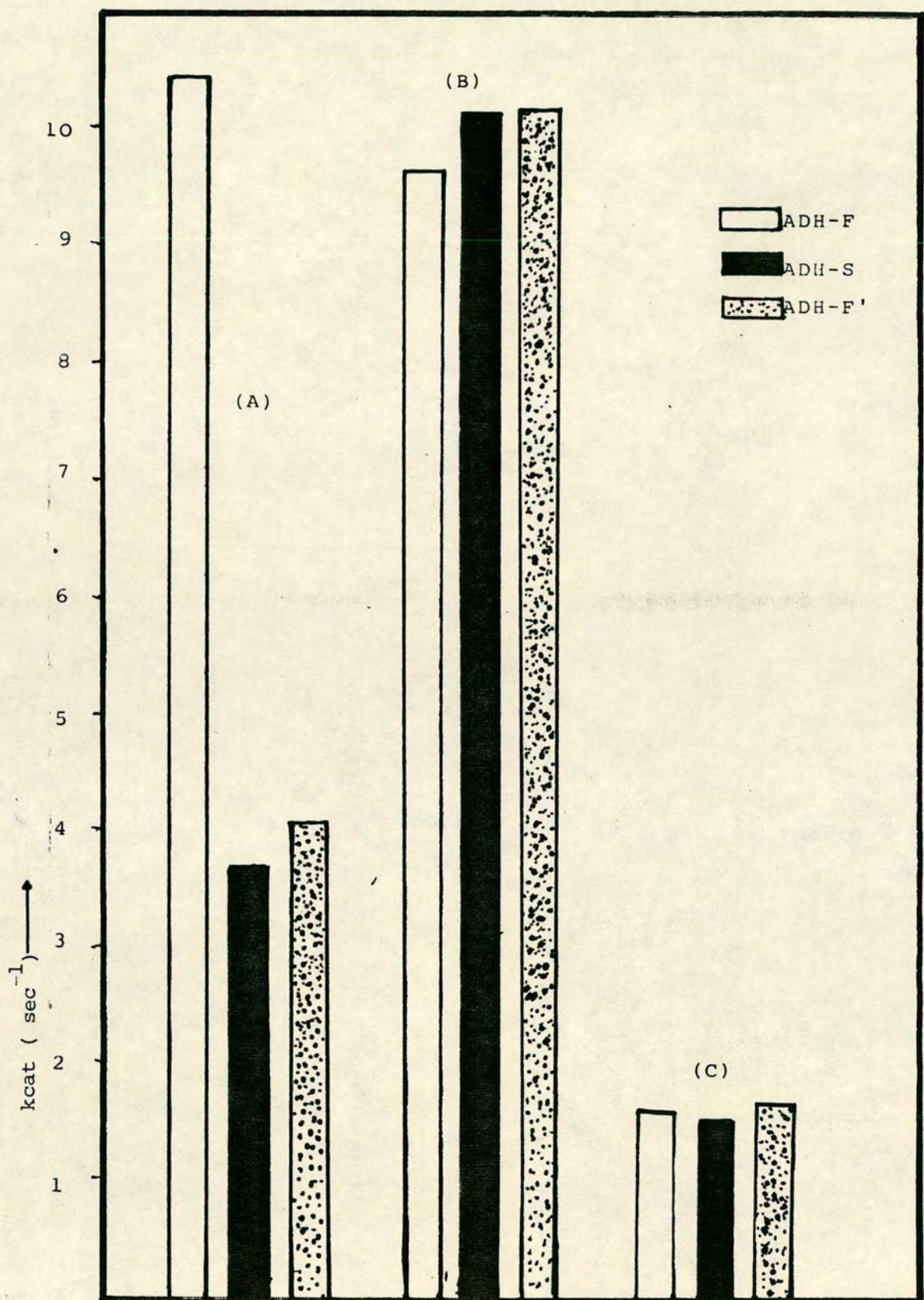


Fig 4.5.b : Graphic representation of k_{cat} for ADH-F, Sand F' using as co-factor (A) NAD⁺, (B) Thio-nicotinamide adenine dinucleotide ; (C) 3-acetyl-pyridine adenine dinucleotide .
(pH 7.45 ; ionic strength 0.1)

replacing the carboxamide group that occupies position 3 of the pyridine ring of the NAD molecule. This group lies next to the reactive site (position 4 of the pyridine ring) of the cofactor. The thionicotinamide adenine dinucleotide (thio-NAD⁺) has a thio-carbamide group in that position while the 3-acetyl pyridine-adenine dinucleotide (3AcPyAD) has an acetyl group (complete formulas are shown in Fig. 4.5.a).

The experiments were conducted in pH 7.45 (buffer composition in Table 2.3) and ionic strength of 0.1 M. Estimates for K_m (app), k_{cat} and k_{cat}/K_m (app) are summarised in Table 4.2. They show that with both the NAD⁺-analogs as cofactors, the difference in catalytic efficiency (k_{cat}) recorded for NAD⁺ between ADH-F and ADH-S disappears. Especially with thio-NAD⁺, k_{cats} for both these variants are very similar with the one recorded for ADH-F with NAD⁺ as the cofactor (Fig. 4.5.b shows graphically the k_{cat} of the variants as recorded with NAD and NAD analogs).

The apparent Michaelis constant (K_m) for the ADH-S enzyme is lower than the K_m (app) of the ADH-F variant. The difference, although not statistically important, persists in the K_m (app) values for thio-NAD⁺, and it has also been reported by Day et al (1974).

Affinity for 3-acetyl-pyridine-adenine dinucleotide is low compared to the one showed for NAD⁺ and thio-NAD⁺. Saturating concentrations of this analog lead to substrate inhibition as can be seen from the reciprocal linear plots (Appendix 2).

4.4 Thermal denaturation of ADH-F'

The thermal denaturation experiments on ADH-F' were an extension of the experiments performed on ADH-S, ADH-F, ADH-D and ADH-N5 by Thatcher and Sheikh (1981). Buffers and conditions were duplicated and the same apparatus was used (section 2.4).

Thermal denaturation^{was} recorded as an increase in absorbance at 280 nm and it is due to aggregation. It is independent of protein concentration above 50 $\mu\text{g/ml}$ but depends on the rate of heating (Thatcher and Sheikh, 1981). Fast heating rates mask the differences between F and S but when slow rates are employed these differences become apparent.

The melting temperature for the F' enzyme is 48°C at a heating rate of 7°C/min. At 1°C/min the melting temperature is 45.5°C. Both estimates are very similar to the ones published by Thatcher and Sheikh (1981) for the S enzyme. In both experiments ADH concentrations were in excess of 50 $\mu\text{g/ml}$.

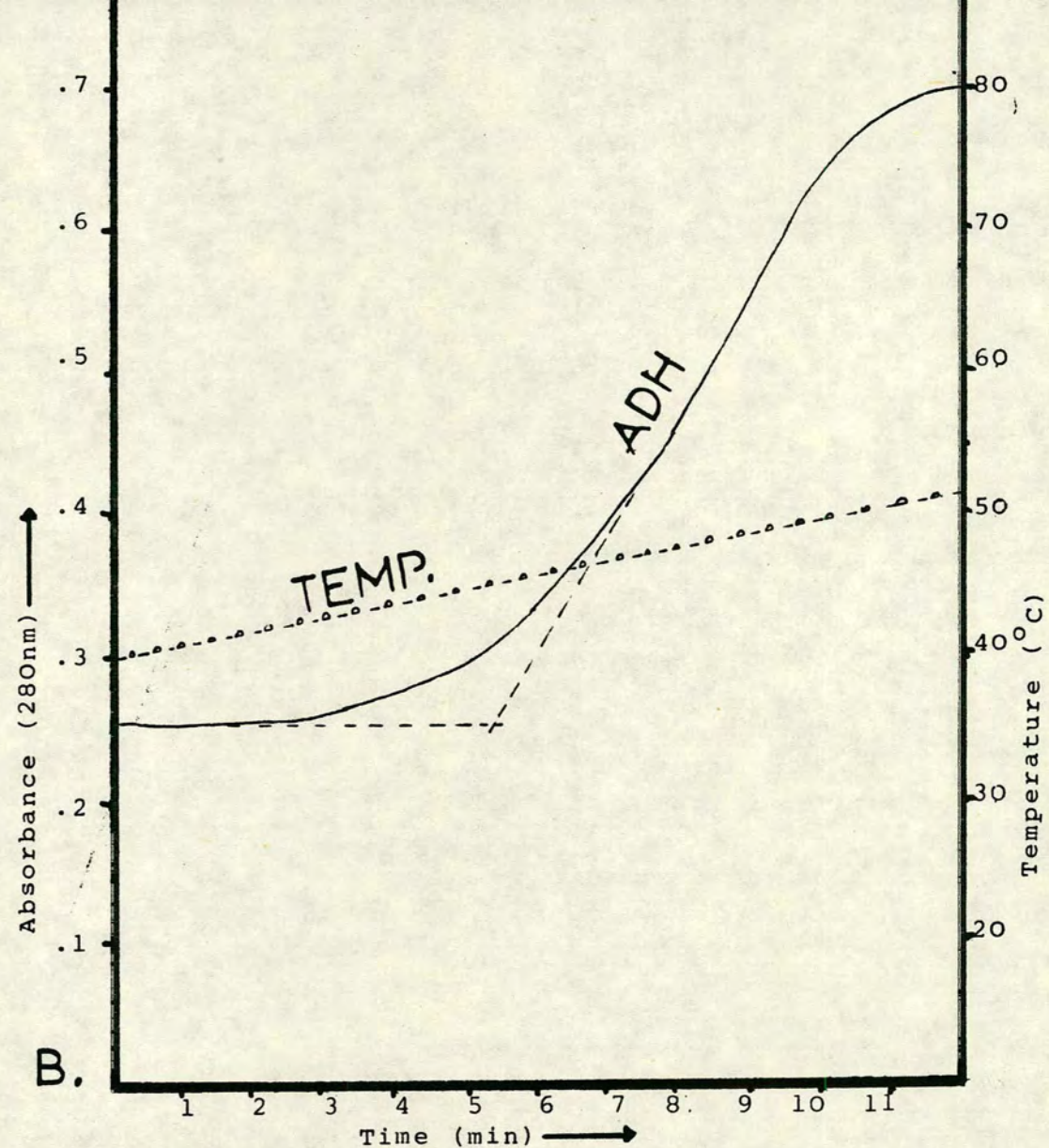
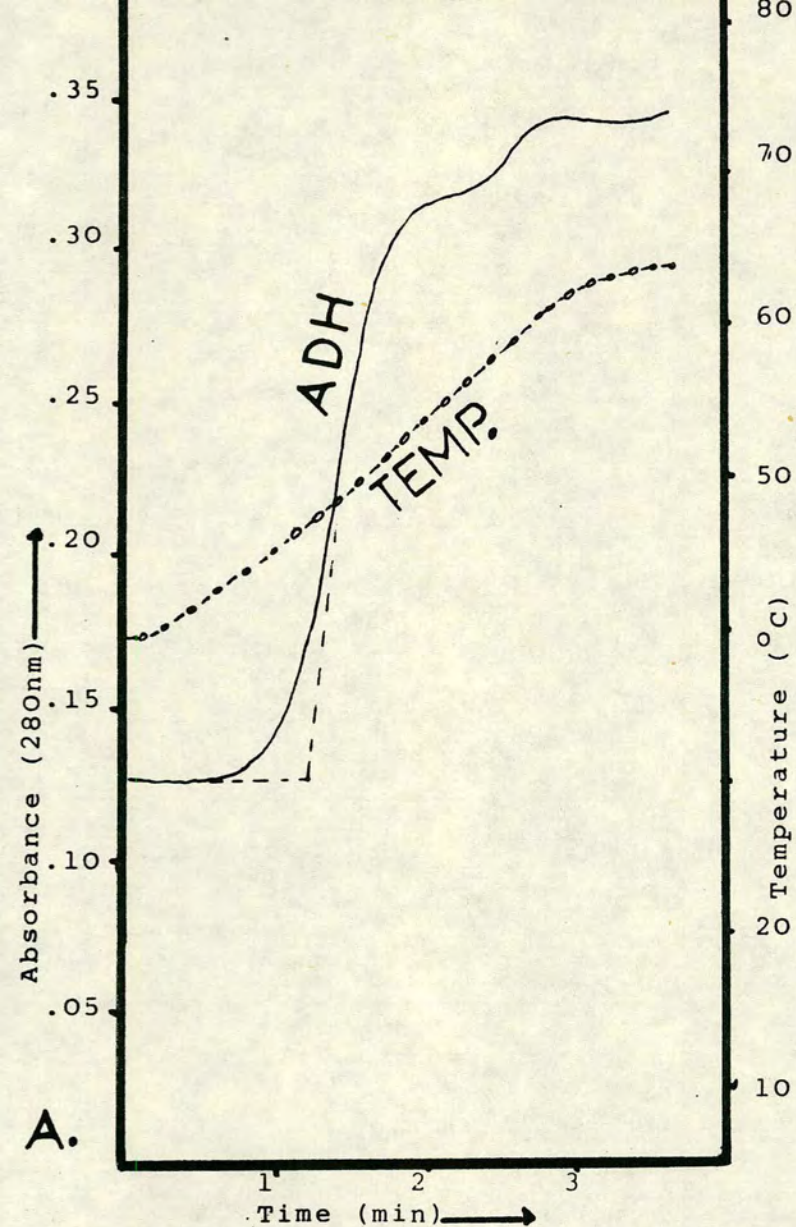


Fig4.6. : Thermal denaturation of Drosophila ADH-F' (ADH-5)
 (a) Heating rate $7^{\circ}\text{C}/\text{min}$ (b) Heating rate $1^{\circ}\text{C}/\text{min}$

CHAPTER 5 Discussion

The aim of this work has been the extensive structural investigation of two *Drosophila* ADH variants (F' and US) and the definition of the changes in conformational stability and enzymatic properties that might have resulted from any substitution(s) detected. The definition of such differences is helpful in two respects, which are in a sense interconnected. On one hand useful information about essential and non-essential residues can be gathered and on the other hand the detailed knowledge of the enzyme and the differences among the variants are necessary if a clear conclusion is to be reached about the forces that maintain allelic frequencies in natural populations.

A high proportion of the amino acid sequence of the ADH-F' enzyme has been completely defined and there is less definitive information about much of the remainder (Fig. 3.13). The only difference revealed between ADH-S and ADH-F' is the alanine → glutamic acid substitution at position 51.

The change in electrophoretic mobility due to this substitution (fractionally different than the one displayed by ADH-F) cannot be easily accommodated by the charge state model proposed by Ohta and Kimura (1975) and King and Ohta (1975). In this model it is assumed that substitutions involving charged residues result in unit charge differences which are the only ones that /

that electrophoretic methods can detect. Earlier, Johnson (1974a) had criticised the creation of such a model by stating that the pKas of ionising side chains are too variable to allow any two charge changes to result in identical changes in net charge in the pH values employed by electrophoresis.

The study of variants in the *Drosophila melanogaster* ADH system showed that, as usual, the truth may lie somewhere inbetween these above mentioned theoretical considerations. The mutation of neutral amino acid residues on the surface of ADH-F to acidic in the case of ADH-UF (Ala 45→Asp), ADH-D (Gly 32→Glu) and ADH-N11 (Gly 14→Asp) resulted in enzymes that are electrophoretically indistinguishable, although the substitutions involve both glutamic and aspartic acid and they are located in different parts of the molecule. These variants comprise an electromorph class as predicted by the charge state model.

But in order to accommodate in this model the rare electrophoretic mobility displayed by ADH-F' we must assume that some conformational changes have occurred due to the presence of lysine 192 (in ADH-S and ADH-F') and glutamic acid-51 (ADH-F' only). The latter possibility can be discounted as experiments reported in Chapters 3 and 4 showed that in specific activity, kinetic constants and conformational stability the ADH-F' enzyme is similar to ADH-S. Chambers et al (1981) considering specific activities and substrate preference reached the same conclusion.

Apart /

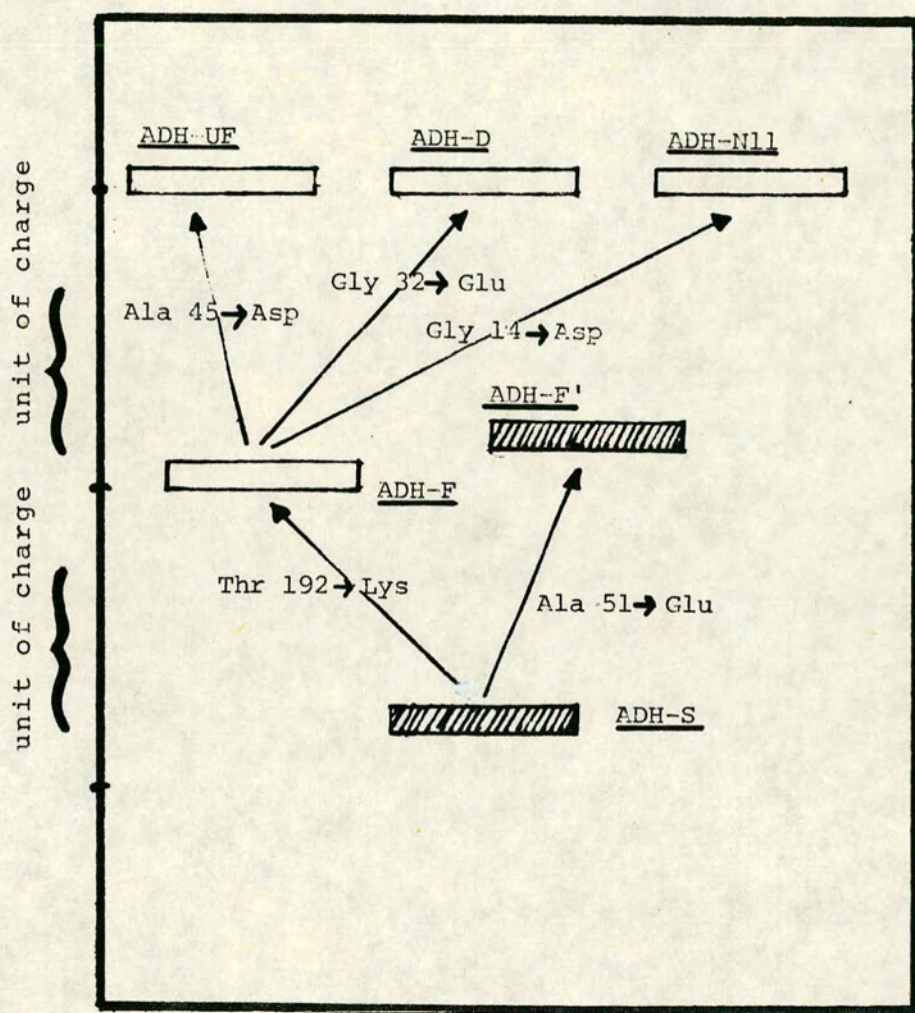


Fig. 5.1 : Charge state and amino acid substitutions of ADH variants derived from either the F or S sequence.

Apart from the enzymatic differences, the threonine-192 (ADH-F)→lysine (ADH-S) substitution must be responsible for conformational re-arrangements in the ADH molecule. The fact that the conformation of the alleloenzymes is dissimilar has been convincingly demonstrated by Thatcher and Sheikh (1981) who, working with highly purified preparations, reported differential response for the variants in a number of test systems. Discrepancy in kinetic properties, for example the disparity in substrate preference reported by Day et al (1974a) and Chambers et al (1981), might also reflect conformational changes that could affect the pKas of several ionisable groups and thus result in fractional, rather than unit, charge difference in the net charge of ADH-F and ADH-S enzymes. Therefore, a gain of an acidic residue on the surface of the ADH-S molecule (as in the case of ADH-F') may indeed result in a unit charge difference but it would not fall in the same electromorph class as the ADH-F enzyme (molilities of variants as well as substitutions involved are shown in Fig. 5.1).

As mentioned before, apart from this substitution the rest of the molecule appears to be identical with the complete or partial sequences published of ADH-S extracted from flies belonging to European, African, American and Australian populations (Fletcher et al, 1977; Thatcher, 1980; Benyajati et al, 1981; Chambers et al, 1981). This coincidence of sequence of ADH in such widespread sampling points to the cosmopolitan distribution of the threonine-lysine substitution between ADH-F and S. It also reinforces the

conclusions

conclusions reached by Sampsell (1977), Kreitman (1980) and McKay (1981) that the level of electrophoretically silent substitutions in *Drosophila melanogaster* is very low. Two such substitutions have been so far detected, one being present in the ADH-UF variant in position 8 where asparagine has mutated to alanine. The second one (Pro 214 → Ser) has been recently reported in a rare variant (ADH-FCh.D) encountered in Australian populations (Chambers et al, 1981).

The alanine glutamic acid substitution in position 51 is inside the presumed NAD^+ binding domain, identified by Thatcher and Sawyer (1980) and Benyajati et al (1981) by secondary structure prediction, using the Chou and Fasman method (Fig. 1.4).

Both these groups put position 51 in the C-terminal end of the helix α -C, which is connected with a short loop with sheet β -D. Both alanine and glutamic acid are strong helix forming amino acids and no secondary structure disruption is expected to result from such a substitution. It was also not expected to have any effects on the adenine binding pocket. This pocket, consisting of hydrophobic residues (9 to 19), has been identified from the investigation of the ADH-N 11 mutant (Thatcher and Retzios, 1981). The similarity shown in catalytic properties between ADH-F' and ADH-S (Chapter 4) was consistent with such expectations. The NAD^+ -analog results showed that no disruption occurred in the nicotinamide binding pocket compared with ADH-S (both analogs have modified nicotinamide moieties) but it could be argued that a greater number of /

of these analogs might be required for a definitive answer.

Another clue that no disruption in secondary or quaternary structure (sub-unit interaction) occurred as a result of the ADH-F' substitution was hinted by the heat denaturation experiments. In these the estimated values for the ADH-F' "melting temperatures" either in quick or slow heating were almost identical with the ones recorded by Thatcher and Sheikh (1981) for the ADH-S enzyme.

7°C/min	1°C/min
ADH-S : 48.0°C	ADH-S : 45.0°C
ADH-F' : 48.0°C	ADH-F' : 45.5°C
(ADH-F : 48.0°C)	(ADH-F : 42.5°C)

However, the substitution, as yet unidentified, responsible for the electrophoretic mobility and enzymatic properties of ADH-US has affected adversely the stability of the molecule, at least in the conditions employed for its purification. Although it has been established that lysine is present in position 192 (Chapter 3), Chambers et al (1981) found that in crude extracts the specific activity and substrate preference place the US enzyme just outside the S-like cluster of ADH variants. Such a result might be due to the instability of the molecule and its readiness to fall to less active forms, a characteristic that made it impossible to be purified by affinity methods.

The results of the kinetic experiments, apart from showing that ADH-F' is kinetically similar to ADH-S, provided some information about /

about the function of the enzyme and the way it is affected by the threonine 192→lysine substitution which is responsible for the detected differences in properties between ADH-F and S.

As can be seen in Fig. 4.3 both ADH-F and S enzymes exhibit similar apparent Michaelis constants at pH 6.5. Moving to lower pH this kinetic constant seems to increase for both alleloenzymes, signifying a drop in affinity for isopropanol. This increase is consistent with a group of pKa around 6.

The decrease in affinity for isopropanol at this part of the pH range is consistent with an imidazole group of a histidine side chain in the mechanism for catalysis in *Drosophila* ADH. The pKa values of imidazole groups, essential for activity, range from between 5 to 8 in enzymes investigated so far (Tanford, 1962; Tanford and Roxby, 1972). This imidazole group must be involved in the binding of alcohol to the enzyme - NAD^+ complex.

This pH dependency of the Michaelis constant for isopropanol complements the results obtained by chemical modification of the histidine residues in *Drosophila* ADH (Thatcher, 1981). Such modification, involving reaction with ethoxyformic anhydride (diethyl pyrocarbonate), inactivated totally the enzyme. The results though are not as straightforward as the ones obtained by Holbrook and Ingram (1973) for lactate dehydrogenase (LDH). Two histidine side chains appear to be modified, but inactivation seems to follow the modification of only one of these (Thatcher, personal communication). In contrast, ethoxyformic anhydride modification of /

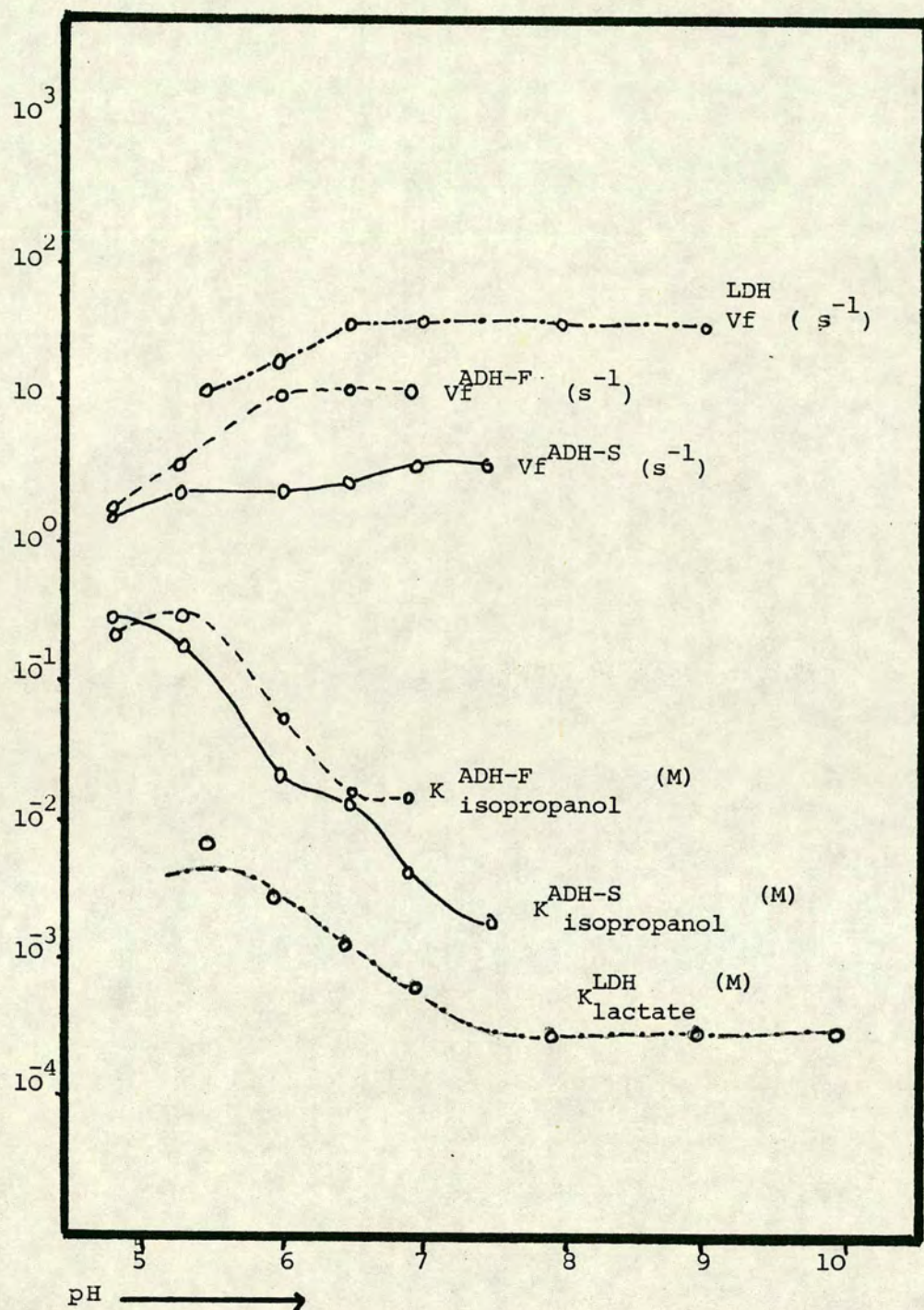


Fig. 5.2. : Comparison of values for the maximum rate of the forward reaction and Michaelis constant for isopropanol of ADH-F and S with the ones recorded by Schwert et al(1967) for beef H4 LDH with lactate as the substrate . (Ionic strength of solutions employed by Schwert et al was 0.2) .

of histidines in LADH results in rapid activation at first, followed by slower inactivation (Morris and McKinley-McKee, 1972).

Another clue pointing to the involvement of an imidazole group in the catalytic mechanism is the similarity in pH dependencies for the forward reaction between the results reported here and the ones published by Schwert et al (1967) for beef H4 LDH. The involvement of histidine in the LDH mechanism has been convincingly demonstrated (Holbrook et al, 1975). Fig. 5.2 shows both sets of results for the forward reaction of these enzymes.

In the group of enzymes related to *Drosophila* ADH in terms of cofactor employed and reaction catalysed (LADH, YADH, LDH, MDH, GAPDH)* the imidazole group of the histidine side chain has been implicated in the LDH and MDH mechanism where it directly binds the substrate to the enzyme-NAD⁺ complex. Histidine is indirectly involved in the yeast and liver ADH where it participates in the binding of the catalytically active zinc-bound water molecule. In the *Drosophila* ADH where no bound zinc has been detected such a role can be discounted. An LDH-like mechanism can be suggested for *Drosophila* ADH (as illustrated in Fig. 5.3) as regards the binding of the alcohol in the active ternary complex. The proposed hydrophobic /

* LADH = liver alcohol dehydrogenase, YADH = yeast alcohol dehydrogenase, LDH = lactate dehydrogenase, MDH = s-malate dehydrogenase, GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

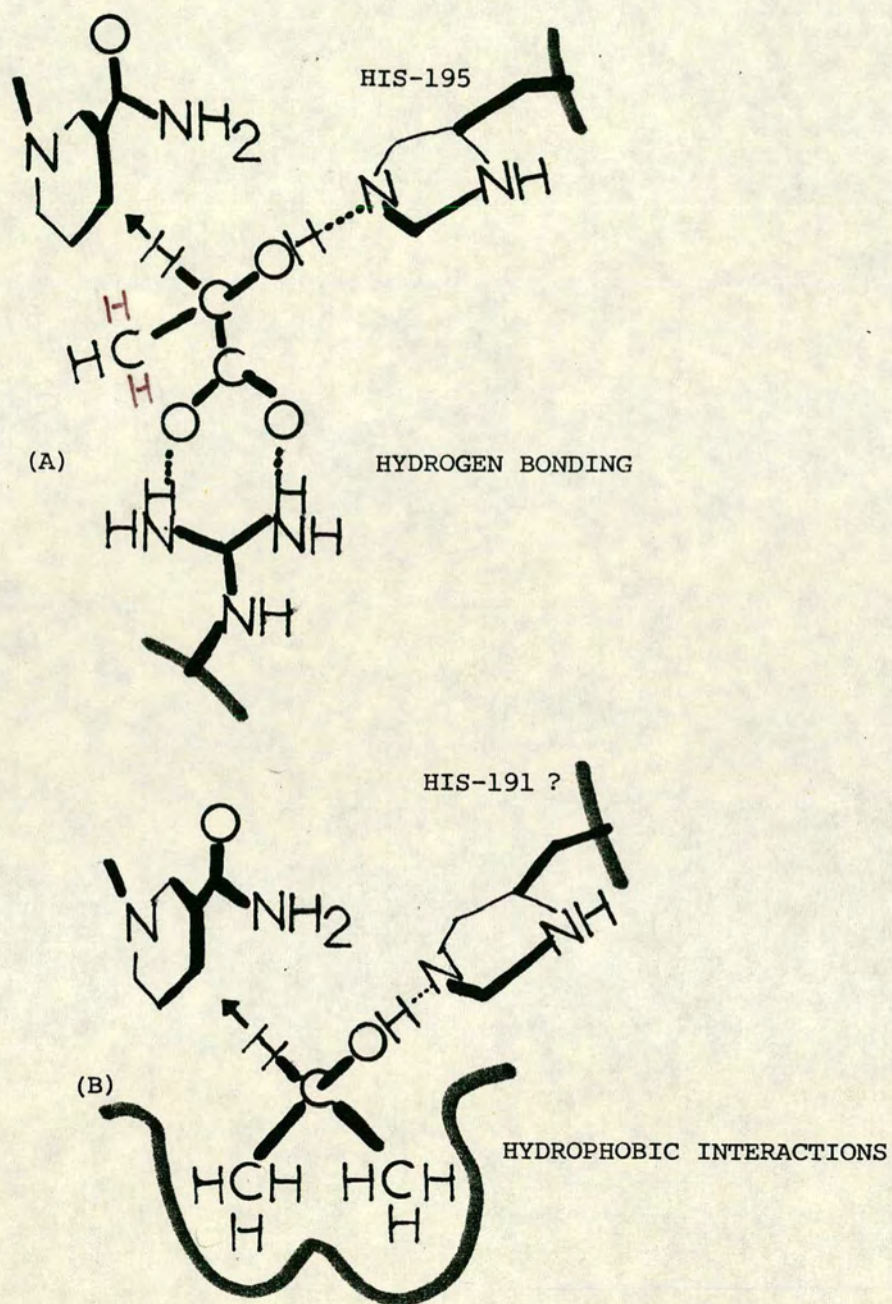


Fig. 5.3 : A. Proposed substrate binding in the ternary intermediate for dogfish M4 LDH (Holbrook et al ,1975)

B. Proposed substrate binding in the ternary intermediate for *Drosophila* ADH .

hydrophobic interactions involved in substrate binding can be postulated from the *Drosophila* ADH preference for secondary and long aliphatic chain alcohols. These hydrophobic interactions must be necessary for the stabilisation of the ternary complex but the substrate preference discrepancy between F and S might be a hint of differences in the hydrophobic pocket geometry of the two alleloenzymes.

An interesting suggestion as to the identity of the presumed catalytically active histidine is provided by the position and environment of such residues in the sequence. From the four (4) histidine residues that are present in the *Drosophila* ADH sub-unit, histidine-250 can be easily discounted as being too close to the C-terminus (only four residues away). Histidine-99 is part of the α -D helix of the detected NAD^+ domain. Histidine-210 is also in an environment of high α -helix forming potential. But histidine-191 is part of a β -sheet forming area resembling strongly the histidine-195 environment of dogfish H4 LDH with its three stranded antiparallel β -sheets. This β -sheet area in *Drosophila* ADH contains a high proportion (40%) of the β -branched residues

*
Dogfish M4 LDH:—(184. β -sheet.194)-(196. β -sheet.209)-(210. β -sheet.214)-
†
Drosophila ADH:-(158. β -sheet.165)-(170. β -sheet.180)-(185. β -sheet.197)-

* Adams et al (1973) † Thatcher and Sawyer (1981)

valine, isoleucine and threonine compared to the sub-unit as a whole (28%). The same is true for the dogfish H4 LDH. Also the histidine /

histidine residue that binds the zinc ion in horse LADH is in a very similar environment.

A definitive answer regarding the identity of the reactive histidine residue may be given by covalent labelling of such a residue with 3-bromoacetyl-pyridine and recovery of the labelled peptide (similar technique was used by Woenkhaus et al (1969) for LDH). Such an experiment must be high on the agenda for further experimentation with *Drosophila* ADH.

Studies with NAD^+ and NAD^+ -analogs (section 4.3) showed that the substitution of the oxygen atom of the carboxamide group of the nicotinamide moiety by sulphur achieves similar activation for *Drosophila* ADH as the Lys-192 \rightarrow Thr substitution. While ADH-F cannot distinguish between NAD^+ and the thio- NAD^+ , ADH-S achieves F-like turnover in the presence of the latter. Surprisingly, apparent Michaelis constants for thio- NAD^+ for both alleloenzymes are almost identical with the ones recorded for NAD^+ , the in vivo coenzyme. The most economic explanation for these results is that the sulphur atom cancels an interaction between Lys 192 and the carboxamide group but both K_m and k_{cat} are derivatives of a number of dissociation constants and a number of changes may have occurred that are not revealed by estimates of these apparent constants. The way the presence of Lys 192 and the oxygen atom of carboxamide group reduce turnover levels in the ADH-S enzyme will be revealed by more detailed kinetic data and by the elucidation of the 3-D structure of both alleloenzymes. But, as the results with ADH-F' showed /

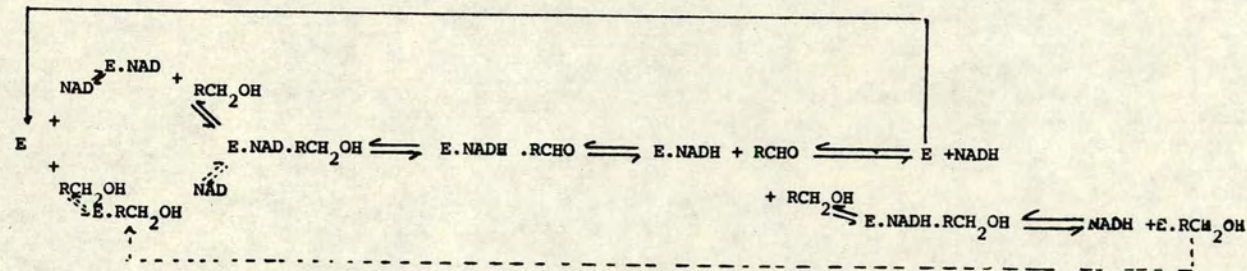


Fig. 5.4. : Reaction scheme proposed by Dalziel and Dickinson (1966) for the explanation of cyclohexanol activation of liver ADH . Pathway shown in dotted lines becomes operational only when the E.RCH₂OH complex is in sufficient concentrations .

showed, the $k_{cat}(\text{NAD}^+)/k_{cat}(\text{thio-NAD}^+)$ ratio can be used to categorise the ADH mutants in F or S-like groups.

Both alleloenzymes showed substrate activation in the presence of saturating concentrations of NAD^+ and isopropanol. Substrate activation under these conditions has been taken as evidence, along with the pH dependencies of kinetic constants (k_{cat} independent of influences in $K_m(\text{app})^{\text{isopropanol}}$), for the existence of a compulsory ordered mechanism where the enzyme-NADH dissociation, that proceeds considerably slower than any other step, is rate determining.

Substrate activation under similar conditions has been demonstrated for liver ADH with cyclohexanol as substrate by Dalziel and Dickinson (1966). These authors explained the phenomenon by assuming the presence of an abortive complex enzyme.NADH.ROH dissociating faster than the enzyme.NADH binary complex. They proposed a scheme (illustrated in Fig. 5.4), based on theoretical considerations, which is an elaboration of the Theorell-Chance mechanism (where ternary complexes are kinetically unimportant). Theorell and McKinley-McKee (1961) advanced a similar explanation for the behaviour of imidazole as an activator of the LADH reaction with large substrate concentrations.

If we compare the sequence, secondary structure prediction and kinetics of Drosophila ADH and other related dehydrogenases such as LADH, YADH, LDH, MDH, GAPDH it can be seen that our enzyme cannot /

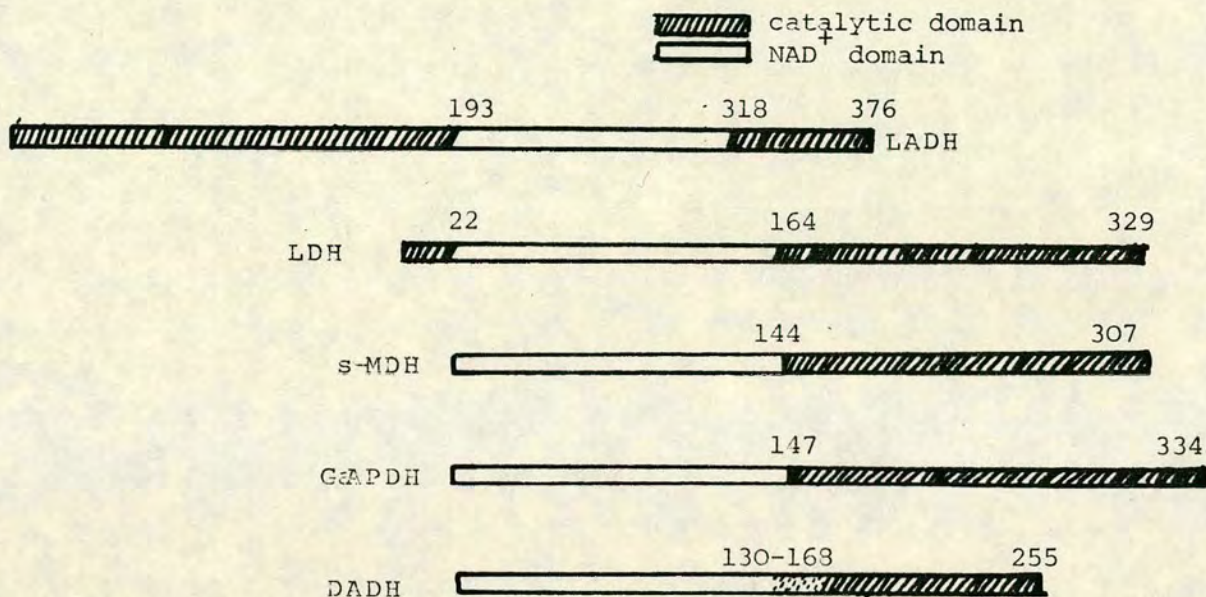


Fig 5.5 : Position and length of the NAD⁺ binding domain of in *Drosophila* ADH (DADH) and related dehydrogenases (Data on LADH, LDH, s-MDH, GAPDH from Rossmann et al, 1975)

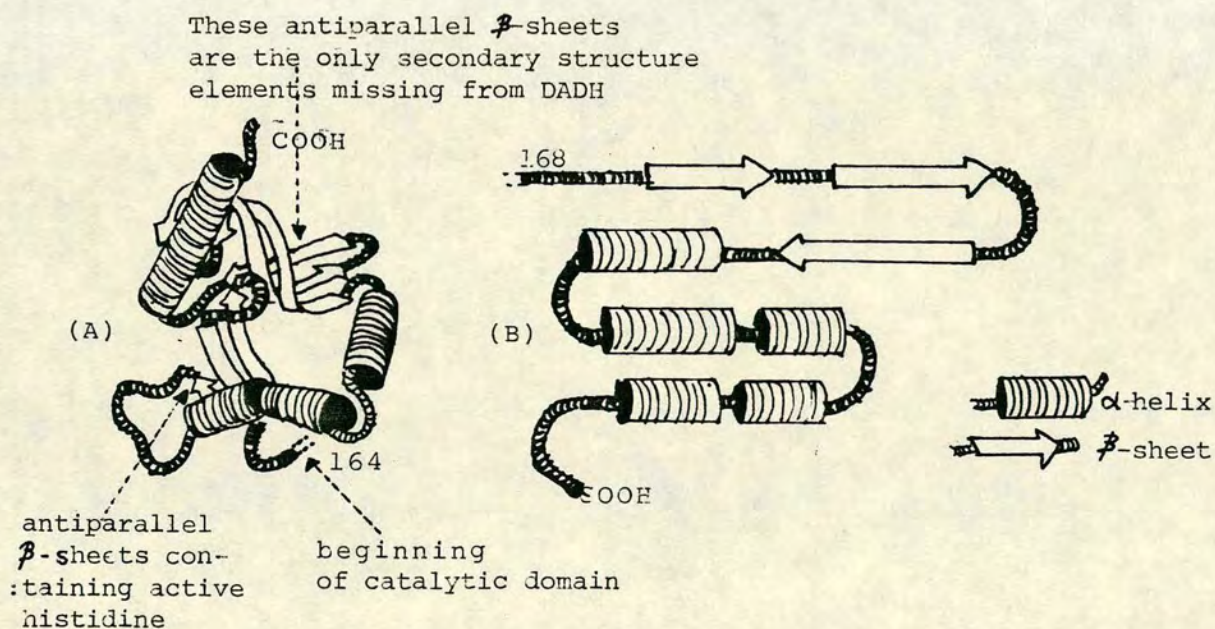


Fig 5.6 : Comparison of the catalytic domain of dogfish H4 LDH with the predicted secondary structure of this domain in *Drosophila* ADH.

- A: catalytic domain of dogfish H4 LDH. (Holbrook et al, 1975)
 B: secondary structure prediction of catalytic domain of *Drosophila* ADH (Thatcher and Sawyer, 1981).

cannot be strictly categorised in any of the existing groups.

For the range of substrates that *Drosophila* ADH acts upon, it resembles the zinc-containing alcohol dehydrogenases (liver and yeast ADH). But this resemblance breaks down when the quantitative preference for primary and secondary alcohols is examined.

In sub-unit structure *Drosophila* ADH definitely resembles lactate and malate dehydrogenases. It is roughly of the same size and the positioning of the NAD^+ binding domain near the N-terminus is similar. In contrast LADH and YADH have a C-terminal situated NAD^+ domain (Fig. 5.5). The similarity with LDH extends to the catalytic domains as well. In dogfish H4 LDH that domain consist of a series of antiparallel β -sheets which contain the active histidine followed by a number of α -helices (α -1G, α -2G, α -3G). The configuration of the domain continues with another series of β -sheets and ends with an α -helix. The catalytic domain of *Drosophila* ADH probably begins at residue 168, where the second of the two introns detected in the gene sequence by Benyajati et al (1981) is situated. From there an area with strong β -sheet forming potential begins, followed by one of strong α -helix forming potential (Thatcher and Sawyer, 1980). Therefore the predicted secondary structure of the *Drosophila* ADH resembles that of LDH apart from the final sequence of β -sheets which is missing (Fig. 5.6). But it must be borne in mind that the *Drosophila* enzyme is 75 residues shorter than dogfish H4 LDH.

Enzymatically the differences with LADH and YADH continue since *Drosophila* ADH does not seem to require zinc or zinc-bound water molecule for activity. But the substrate is held by hydrophobic interactions as in the zinc enzymes.

Thatcher (1981) reported that the thiol group of one of the two cysteine residues in the *Drosophila* ADH sub-unit reacts slowly in the presence of excess 5,5'-dithiobis-(2-nitrobenzoic acid) to produce an inactive enzyme. This reaction is quite unlike the one given by the hyperactive thiol group involved in catalysis in GAPDH (Harris et al, 1963) but similar results have been obtained for LDH (Holbrook, 1966). In dogfish LDH compounds that modify the thiol group of cysteine-165 achieve inactivation by being positioned between residues asparagine-168 and histidine-195 thus inhibiting histidine-195 moving towards the substrate. The area around this cysteine residue is highly conserved in all LDHs studied so far (Holbrook et al, 1975). In *Drosophila* ADH the sequence around cysteine-135 bears strong resemblance to this conserved LDH section (Fig. 5.7). A further sequence homology has been demonstrated in the adenine binding site (Thatcher and Retzios, 1981) where *Drosophila* ADH has retained a number of residues invariable in the sequences of LDH, GAPDH, GDH, LADH and YADH for this site (Fig. 5.8).

As far as the quaternary structure is concerned, active *Drosophila* ADH is a dimer, like MDH and LDH. LDH is a tetramer (it may be a dimer in lower organisms like the horseshoe crab and *E. coli*, /

Chicken H4 LDH	Val	Ile	Gly	Ser	Gly	Cys	Asn	Leu	Asp	Thr	Ala	-
Pig M4 LDH	Val	Ile	Gly	Ser	Gly	Cys	Asn	Leu	Asp	Ser	Ala	-
Dogfish M4 LDH	Ile	Ile	Gly	Ser	Gly	Cys	Asn	Leu	Asp	Ser	Ala	-
Drosophila ADH	Pro	Gly	Gly	Ile	Ile	Cys	Asn	Ile	Gly	Ser	Val	-

Fig. 5.7 : Similarities of the sequence around Cys-135 of Drosophila ADH with a highly conserved sequence segment of all LDHs studies so far.
(data on chicken, pig and dogfish LDHs from Holbrook et al, 1975)

	β A			α B										β B																											
LDH	21	NK	ITVV	G	V	G	A	V	G	M	A	C	A	I	S	I	L	M	K	D	L	A	D	E	V	A	L	V	D	V	M	E	D	K	L	K					
GAPDH	2	S	K	I	G	I	D	G	-	F	G	R	I	G	R	L	V	L	R	A	A	L	S	C	G	-	A	Q	V	V	A	V	N	D	P	F	I	A	L	E	Y
GDH	219	K	R	V	A	L	S	G	-	S	G	N	V	A	Q	Y	A	A	L	K	L	I	E	L	G	-	A	T	V	V	S	L	S	D	S	K	G	A	L	V	A
LADH	193	S	T	C	A	V	F	G	-	L	G	G	V	G	L	S	V	I	M	G	C	K	A	A	G	-	A	A	R	I	I	G	V	D	I	N	K	D	K	F	A
YADH	171	H	W	V	A	I	S	G	A	A	G	G	L	G	S	L	A	V	Q	Y	A	K	A	M	G	-	-	Y	R	V	L	G	I	D	G	G	E	G	K	E	E
DADH	8	N	V	I	F	V	A	G	-	L	G	G	I	G	L	O	T	S	K	Q	L	L	K	R	D	-	L	K	N	L	V	I	L	O	R	I	E	N	P	A	A

Fig. 5.8 : Alignment of the sequence and the observed secondary structure of the adenine ribose binding sub-domain of LDH (Rossman et al, 1971), LADH (Eclund et al, 1976), GAPDH (Moras et al, 1975) with the sequence and predicted secondary structure of this sub-domain in GDH (Wootton, 1974) and DADH (Thatcher, 1980).

(from Thatcher and Retzios, 1980)

E. coli, according to Selander and Young, 1970). The quaternary structure of MDH, LDH and GAPDH shows the existence of a Q-axis, in contrast to LADH. This axis (defined by Rossmann et al, 1973) is of use comparatively for dehydrogenases, since in the cases where it is known to be present the sub-units associate by contacts of the NAD^+ binding domain. These contacts consist of interactions between α -C, α -B and α -3G helices in one sub-unit with α -3G, α -B and α -C of the other. However the substitution in ADH-F' argues against the existence of this axis in *Drosophila* ADH. Glutamic acid-51 in this variant is placed by secondary structure prediction in the α -C helix area, replacing the alanine present in ADH-S and ADH-F. The fact that no discernible destabilisation has occurred by the insertion of this charged residue with different side-chain geometry is strong evidence that this area is not involved in sub-unit interactions, which depend to a large extent on van der Waals contacts and hydrogen bonds and presuppose complementary surfaces. Further confirmation is provided by the Ala 45 \rightarrow Asp substitution on the same helix that exists in the functional F-like variant ADH-UF (Thatcher, 1980).

These considerations tend to put *Drosophila* in a category of its own inside the NAD^+ dependent dehydrogenase family. In the evolutionary tree of present day dehydrogenases suggested by Buehner et al (1973), it should therefore be placed independently before the Q-axis formation and after the reversal of the NAD^+ binding domain (Fig. 5.9). Final confirmation must of course await crystallographic results. /

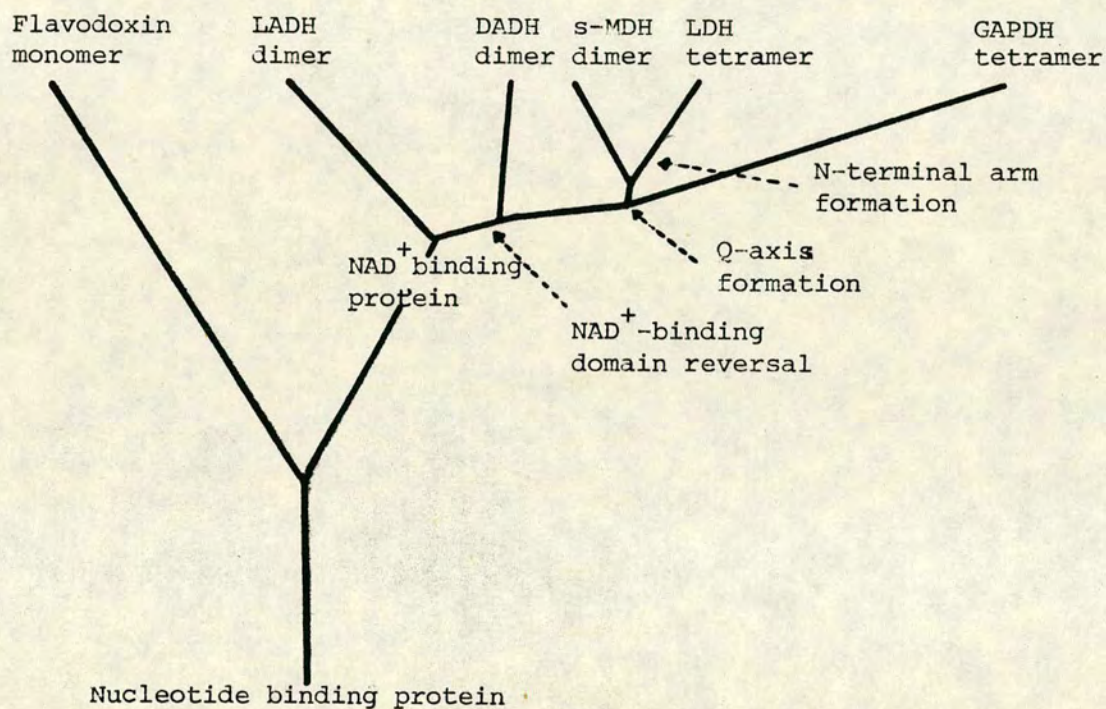


Fig. 5.9 : Evolutionary tree showing the divergence of dehydrogenases and flavodoxin according to Buehner et al (1973), incorporating *Drosophila* ADH.

await crystallographic results.

It is tempting, for a molecular biologist, to try to understand how the observed properties of the enzyme in general and its alleloenzymes in particular influence interspecific and intraspecific selection respectively.

Besides ADH-F', several ADH variants (either naturally occurring or chemically induced) have been investigated (Table 5.1).

ADH-S	(1)	Lysine (192)
ADH-F	(2)	Lysine (192) → Threonine
ADH-UF	(3)	Lysine (192) → Threonine
		Asparagine (8) → Alanine
		Alanine (45) → Aspartic acid
ADH-D	(4)	Lysine (192) → Threonine
		Glycine (232) → Glutamic acid
ADH-F'	(5)	Alanine (51) → Glutamic acid
Table 5.1. Divergence of the primary sequence of ADH-variants from the primary sequence of ADH-S.		
(1) & (2)	Fletcher et al (1978), Thatcher (1980), Benyajati et al (1981).	
(3)	Camfield and Thatcher (1977), Thatcher (1980).	
(4)	Schwartz and Jörnvall (1976).	
(5)	This thesis.	

The neutralist theorists proposed that if natural selection /

selection is acting on allelic frequencies at all, it is acting solely on differences in charge state (Ohta and Kimura, 1975) or number of amino acid substitutions (King and Ohta, 1975) - discussed in section 1.1. Such selection is of course unrelated to any environmental factors and differences in enzymic or conformational properties are not expected to be under selection. But if this were true, ADH-F' frequencies should be far higher than those of ADH-UF which is much more removed from both common alleles (F and S) in charge state (and number of substitutions, although a small part of F' sequence is as yet undetermined). However, this is not the case (David, private communication).

Chambers et al (1981) investigated several properties (thermostability, specific activity, isopropanol/ethanol activity ratio) in a number of ADH variants. On this basis they concluded that all ADH variants can be categorised into two functional groups: the F-like and S-like, from the most common enzyme type of each found in natural populations. The identity of ADH-F' properties with those of ADH-S detected in the experiments reported in this thesis, confirms the validity of such grouping. These functional classes can be correlated with the identity of the residue occupying position 192. When lysine is present they are S-like, when threonine F-like.

Neutralist-type selection is inconsistent with observations in the ADH-locus, but selection acting on the functional differences has only been able to distinguish the two above mentioned classes

The /

The individual members of these classes should therefore be neutral with respect to one another, and the frequency of the rare variants in a given population must be a function of the mutation rate and the frequency of the common allele they are derived from in that population.

But how does selection act on the functional characteristics of *Drosophila* ADH? Interspecifically, levels of ADH activity in *Drosophila melanogaster* are significantly higher than the ones displayed by several other *Drosophila* species like *Drosophila simulans* that happens to be a co-patric species. These higher levels of ADH activity, as has been conclusively shown, confer an advantage to the *melanogaster* fly in ethanol-rich environments (up to 10%), either in laboratory controlled conditions or in the natural habitat (section 1.2).

It is therefore puzzling that several of its properties argue against any direct involvement of the enzyme in alcohol metabolism. For one thing, its substrate preference is "wrong". It is most active with secondary and cyclic alcohols, which do occur in environmental pools of ethanol and other primary alcohols. Such substrates lead to the formation of ketones, substances much more toxic than the alcohols they replace. In order to resolve this difficulty Anderson and MacDonald (1981) suggested that the less active ADH isoenzymes (ADH-3, ADH-1) are results of a regulatory mechanism. In such a mechanism a NAD^+ -isopropanol addition complex reacts with the ADH-5 sub-unit, inactivating it and thus preventing ketone /

ketone poisoning. But such a reaction is slow and virtually irreversible and must be a blunt and wasteful instrument of a regulatory mechanism. Furthermore, while it is known that ADH is inhibited by aldehyde (also more poisonous than alcohols), no connection with the aldehyde oxidase (Ald.ox) has been demonstrated. On the contrary, Ald.ox⁽⁻⁾ flies show the same level of ethanol tolerance as Ald.ox⁽⁺⁾ flies (David et al, 1978). Increases in ethanol tolerance have not been convincingly correlated with increases in the frequency of the most active allele (ADH-F) but rather the reverse (Gibson, 1979). It must be also remembered that alternative pathways for ethanol oxidation do exist (Walsh, 1973) and could be employed by *Drosophila*.

It is therefore important to inquire into the chemical basis of alcohol tolerance. Where mechanisms of alcohol poisoning have been investigated, it has been shown that a major effect is membrane dissolution. Experimental animals develop tolerance, not by directly acting on the agent but by reinforcing the membranes with sterols. Such a response is a multi-step one and involves a number of enzymes. David and Bocquet (1977) showed that the ethanol tolerance in *Drosophila* is also accomplished by a multi-step process where ADH activity levels play a rather limited role. Could then *Drosophila* ADH be connected with sterol (or steroid) metabolism? In horse liver ADH one of the two isoenzymes (isoenzymes E and S are coded by different genes but are broadly similar in structure and amino acid composition) has, on the evidence of substrate specificity, a marked preference towards certain /

certain steroid side-chain hydroxyl groups (Okuda and Takigawa, 1970; Björkhem et al, 1973). The involvement of ADH in steroid metabolism will account for the discrepancies between its properties and its physiological role. Such a hypothesis will also explain the anomalous result reported by Bijlsma-Meeles and van Delden (1974). These authors, examining tolerance in ethanol rich environments recorded a statistically significant increase in ADH-F frequencies with methanol which is not a substrate but is a membrane solvent.

Attention has also justifiably been focused on selection at the intraspecific level. A number of mechanisms for the maintenance of the polymorphism in the ADH locus have been suggested. Most of these assume that alcohols are the environmental parameters responsible for selection and that the enzyme is an active detoxification agent. Such mechanisms (reviewed in section 1.2) are unsatisfactory for the reasons mentioned above. However, evidence that connects ADH allele frequencies with means or extremes of temperature is more convincing. Both Pipkin et al (1973) and Wilks et al (1980) showed a significant positive correlation between extreme minimum and mean temperatures and ADH-alleles frequencies. This was unexpected because most researchers assumed that temperature, as the selective agent, would have tended to act on the thermostability differences between the two alleloenzymes. But extreme maximum temperatures and allele frequencies were not correlated. These results agree well with the fact that variants that show increased thermostability, compared to the common enzyme type they are derived from, do not seem to be under selection (Sampsell, 1977).

The way that low temperatures can favour the F allele is not easy to see. But work in temperature adaptation (Das, 1965; Prosser, 1967) has suggested that high enzymic activity is favoured in poikilotherm organisms adapted to cold conditions. ADH-F, we may then conclude, will be favoured in colder climates. But examining the $k_{cat}/K_m(\text{isopropanol})$ ratios for both alleloenzymes (Fig. 4.4) we can see that such a conclusion cannot be correct. In physiological pH (around 7.0) the ratio is the same for both F and S and if we assume that there are low concentrations of the substrate, and that such concentrations occur in vivo, there is little to choose between the variants. The lower turnover of the S enzyme is compensated by correspondingly lower K_m for the substrate. In fact in low concentrations (<1 mM) ADH-S is better and such superiority increases as the pH shifts even fractionally towards basic values. It must also be remembered that ADH-S has lower K_m values for NAD^+ although probably not significantly so. Thus it is only between the pH values 6.2 to 6.8 that ADH-F is kinetically superior in rate limiting concentrations of the substrate. Between 6.5 and 6.9 ADH-S is weak enzymatically while ADH-F achieves its highest values.

But how can temperature influence the pH values of ADH environment in the flies? Some clues are provided by the strategies employed by poikilotherms in order to increase body temperature in cold conditions. Such strategies involve the contraction of the muscles and tracheoids. With limited air intake anaerobic metabolites may reduce pH values of intracellular or intercellular environment. /

environment. It is well known that in muscles haemoglobin notices and responds to such an effect.

These considerations suggest a form of balancing selection for the maintenance of the ADH polymorphism in *Drosophila melanogaster*. In areas where it is more probable for the fly to encounter low temperatures while it is active the ADH-F allele will be selected for. Outside these areas it will be selected against, and flies will tend to be monomorphic for ADH-S.

Although such a mechanism is consistent with much of what is known about the ADH locus and what is presented here, detailed information about the environmental factors that influence the niche of *Drosophila melanogaster*, its temporal and geographical extent and the precise role of the enzyme in the physiology of the fly must be forthcoming before final conclusions are drawn.

B I B L I O G R A P H Y

- M. Adams, M. Buehner, K. Chandrasekmar, G. Ford, M. Hackert, A. Liljas, M. Rossmann, I. Smiley, W. Allison, J. Everse, N. Kaplan and S. Taylor (1973).
Proc. Natl. Aca. Sci. U.S.A. 70:1968.
- G. Allen (1981) "Sequencing of protein and peptides",
Elsevier/North Holland: Amsterdam, New York and Oxford.
- R. Ambler (1963). Biochem. J. 89:349.
- R. Ambler (1981). In: "Amino acid analysis" pp 119-137
ed. J. Rattenbury, Ellis Horwood, Chichester.
- S. Anderson and J. MacDonald (1981). Biochem. Genet. 19:421.
- A. Auffret, D. Williams and D. Thatcher (1978) FEBS Letts 90:324.
- F. Ayala, J. Powell and T. Dobzhansky (1971).
Proc. Natl. Aca. Sci. U.S.A. 68:2480.
- F. Ayala, J. Powell, M. Tracey, C. Mourao and S. Perez-Salas (1972).
Genetics 71:113.
- F. Ayala, J. Powell and M. Tracey (1972). Genet. Res. Camb. 20:19.
- F. Ayala and M. Tracey (1974). Proc. Natl. Aca. Sci. U.S.A. 71:999.
- F. Ayala, M. Tracey, L. Barr, J. MacDonald and S. Perez-Salas (1974).
Genetics 74:343.
- B. Barnes and A. Birley (1975). Heredity 35:115.
- B. Barnes /

- B. Barnes and A. Birley (1978). Heredity 40:51.
- J. Bearden (1978). BBA 533:525.
- C. Benyajati, A. Place, D. Powers and W. Sofer (1981).
Proc. Natl. Aca. Sci. U.S.A. 78:2717.
- A. Bernstein, L-H. Throckmorton and J. Hubby (1973).
Proc. Natl. Aca. Sci. U.S.A. 70:3928.
- E. Bijlsma-Meeles and W. van Delden (1974).
Nature (London) 247:369.
- A. Birley and B. Barnes (1973). Heredity 31:413.
- A. Birley and B. Barnes (1975). Heredity 35:121.
- I. Björkhem, H. Jörnvall and E. Zeppezauer (1973).
BBRC 52:413.
- M. Bradford (1976). Anal. Biochem. 72:248.
- C-I. Bränden, H. Jörnvall, H. Eklund and B. Furugen (1975).
In: "The Enzymes", Vol XI, pp 104-190, ed. P. Boyer, Academic Press,
New York.
- D. Briscoe, A. Robertson and J. Malpica (1975).
Nature (London) 253:148.
- M. Buehner, G. Ford, D. Moras, K. Olsen and M. Rossmann (1973).
Proc. Natl. Aca. Sci. U.S.A. 70:3052.
- J. Buettner-Janus and R. Hill (1965). In: "Evolving Genes and
Proteins" p 167, ed. V. Bryson and H. Vogel, Academic Press, New York.
- V. Byers, /

V. Byers, D. Lambeth, H. Lardy and E. Margoliash (1971).
Federation Proceedings 30:1286.

D. Cavener and M. Glegg (1978). Genetics 90:629.

G. Chambers, J. MacDonald, M. MacElfresh and F. Ayala (1978).
Biochem. Genet. 16:757.

G. Chambers, A. Wilks and J. Gibson (1981). In preparation.

P. Chou and G. Fasman (1974). Biochemistry 13:222.

M. Ciotti and N. Kaplan (1957). In: "Methods of Enzymology",
Vol III, pp 890, Academic Press, New York.

B. Clarke (1970). Science 168:1009.

B. Clarke (1970). Nature (London) 228:159.

B. Clarke (1975). Genetics 79:101.

B. Clarke, R. Camfield, A. Galvin and C. Pitts (1979).
Nature (London) 280:517.

B. Clarke and F. Allendorf (1979). Nature (London) 279:732.

T. Courtright, R. Imberski and H. Ursprung (1966) Genetics 54:2151

J. Coyne (1976). Genetics 84:593.

J. Coyne, A. Felton and R. Lewontin (1978).
Proc. Natl. Aca. Sci. U.S.A. 75:5090.

K. Crowshaw, S. Jessup and P. Ramwell (1967). Biochem. J. 103:79.

K. Dalziel /

K. Dalziel (1957). Acta Chem. Scand. 11:1706.

K. Dalziel (1963). J. Biol. Chem. 238:1538.

K. Dalziel (1975). In: "The Enzymes", Vol XI, pp 2-61,
ed. P. Boyer, Academic Press, New York.

K. Dalziel and F. Dickinson (1966). Biochem. J. 100:491.

A. Das (1965). Ph.D. Thesis, University of Illinois.

J. David and C. Bocquet (1976). Comp. Biochem. Physiol. 54C:71.

J. David, C. Bocquet, M. Arens and P. Fouillet (1976).
Biochem. Genet. 14:989.

J. David and C. Bocquet (1977). Genetics 47:43.

J. David (1978). La Recherche 9:482.

J. David, C. Bocquet, J. Van Herrewege, P. Fouillet and M. Arens
(1978). Biochem. Genet. 16:203.

T. Day, P. Hillier and B. Clarke (1974a). Biochem. Genet. 11:141.

T. Day, P. Hillier and B. Clarke (1974b). Biochem. Genet. 11:155.

T. Day and L. Needham (1974). Biochem. Genet. 11:167.

D. Dent (1947). Biochem. J. 41:240.

M. Dixon (1953). Biochem. J. 55:161.

R. Dolan and A. Robertson (1975). Heredity 35:311.

G. Dunn, /

G. Dunn, T. Wilson and K. Jacobson (1969). J. Exptl. Zool. 171:185.

M. Eklund, B. Nordsrom, E. Zeppezauer, G. Soderlund, I. Ohlsson,
B-O. Boiwe, O. Tapia and C-I. Bränden (1976). J. Mol. Biol. 102:27.

B. Erlanger and W. Cohen (1969) J. Amer. Chem. Soc. 85:348.
W. Fitch and C. Langley (1976). Federation Proceedings 35:2092.

T. Fletcher, F. Ayala, D. Thatcher and G. Chambers (1978).
Proc. Natl. Aca. Sci. U.S.A. 75:5609.

E. Ford (1940). Ann. Eugen. 10:227.

J. Gibson (1970). Nature (London) 227:959.

J. Gibson and R. Miklovich (1971). Experientia 27:99.

J. Gibson (1979). Aust. J. Biol. Sci. 32:387.

J. Gibson, G. Chambers, A. Wilks and J. Oakeshott (1980).
Aust. J. Biol. Sci. 33:479.

J. Gillespie and K. Kojima (1968).
Proc. Natl. Aca. Sci. U.S.A. 61:582.

W. Gray and B. Hartley (1963). Biochem. J. 89:59p.

W. Gray (1972a). In: "Methods in Enzymology", Vol XXV, pp 121-143,
Academic Press, New York.

W. Gray (1972b). In: "Methods in Enzymology", Vol XXV, pp 333-344,
Academic Press, New York.

E. Grell, K. Jacobson and J. Murphy (1965). Science 149:80.

E. Grell, /

E. Grell, K. Jacobson and J. Murphy (1968).

Ann. N.Y. Aca. Sci. 151:441.

A. Grossman, L. Koreneva and L. Ulitscaya (1970).

Genetica (Soviet) 6:91.

M. Grunow and W. Schöpp (1978). FEBS Letts. 94:375.

B. Gutte, M. Däumigem and E. Wittschrieber (1979).

Nature (London) 281:650.

J. Haldane (1957). J. Genet. 55:511.

H. Harris (1966). Proc. Roy. Soc. B. 164:298.

H. Harris, D. Hopkinson and Y. Edwards (1977).

Proc. Natl. Aca. Sci. U.S.A. 74:698.

J. Harris, B. Meriweather and J. Park (1963).

Nature (London) 198:154.

B. Hartley (1970). Biochem. J. 119:805.

W. Heed (1978). In: "Ecological Genetics - The Interface", p 109,
ed. P. Brussard, Springer-Verlag, New York.

N. Hewitt, S. Pipkin, N. Williams and P. Chakrabarty (1974).

J. Heredity 65:141.

C. Hirs (1956). J. Biol. Chem. 219:611.

J. Holbrook (1966). Biochem. Z. 344:141.

J. Holbrook and H. Gutfreund (1973). FEBS Letts. 31:157.

J. Holbrook /

J. Holbrook and V. Ingram (1973). Biochem. J. 131:729.

J. Holbrook, A. Liljas, S. Steindel and M. Rossmann (1975).

In: "The Enzymes", Vol XI, pp 191-292, Academic Press, New York.

R. Imberski, W. Sofer and H. Ursprung (1968). Experientia 24:504.

K. Jacobson (1968). Science 159:324.

K. Jacobson, J. Murphy and F. Hartman (1970).

J. Biol. Chem. 245:1075.

K. Jacobson and P. Pfuderer (1970). J. Biol. Chem. 245:3938.

K. Jacobson, J. Murphy, J. Knopp and J. Ortiz (1972).

Arch. Bioch. Bioph. 149:22.

F. Johnson and C. Denniston (1964). Nature (London) 204:906.

F. Johnson and A. Powell (1974).

Proc. Natl. Aca. Sci. U.S.A. 71:1783.

G. Johnson (1974^a). Genetics 78:771.

G. Johnson (1974^b). Science 184:28.

G. Johnson (1977). Biochem. Genet. 15:665.

T. Jukes and J. King (1971). Nature (London) 231:114.

A. Kamping and W. van Delden (1978). Biochem. Genet. 16:541.

V. Keil-Dlouha, N. Zylber, J-H. Imhoff, N-T. Tong and B. Keil (1971).

FEBS Letts. 16:291.

M. Kimura /

- M. Kimura and J. Crow (1964). *Genetics* 49:725.
- M. Kimura (1968a). *Nature (London)* 217:624.
- M. Kimura (1968b). *Genet. Res., Camb.* 11:247.
- M. Kimura (1969). *Proc. Natl. Aca. Sci. U.S.A.* 63:1181.
- M. Kimura and T. Ohta (1971). *Nature (London)* 229:467.
- J. King and T. Jukes (1969). *Science* 164:788.
- J. King and U. Laemmli (1971). *J. Mol. Biol.* 62:456.
- J. King and T. Ohta (1975). *Genetics* 79:681.
- J. Knopp and K. Jacobson (1972). *Arch. Bioch. Bioph.* 149:36.
- K. Kojima and K. Yarbrough (1967).
Proc. Natl. Aca. Sci. U.S.A. 57:645.
- K. Kojima and Y. Tobari (1969). *Genetics* 61:201.
- M. Kreitman (1980). *Genetics* 95:467.
- C. Langley and W. Fitch (1973). In: "Genetic Structure of Populations", pp 246-262, ed. N. Morton, University Press of Hawaii, Honolulu.
- C. Langley and W. Fitch (1974). *J. Mol. Biol.* 3:161.
- A. Leigh Brown and C-Y. Lee (1979). *Biochem. J.* 179:479.
- N. Lewis and J. Gibson (1978). *Biochem. Genet.* 16:159.
- R. Lewontin /

- R. Lewontin and J. Hubby (1966). *Genetics* 54:595.
- R. Lewontin (1974). "The Genetic Basis of Evolutionary Change",
Columbia University Press, New York.
- H. Lineweaver and J. Burk (1934). *J. Am. Chem. Soc.* 56:658.
- D. Lowry, M. Rosebrough, A. Farr and R. Randall (1951).
J. Biol. Chem. 193:265.
- J. MacDonald and J. Avise (1976). *Biochem. Genet.* 14:347.
- J. MacDonald, G. Chambers, J. David and F. Ayala (1977).
Proc. Natl. Aca. Sci. U.S.A. 74:4562.
- J. MacDonald and F. Ayala (1978). *Genetics* 89:371.
- J. MacDonald, S. Anderson and M. Santos (1980). *Genetics* 95:1013.
- J. McKay (1981). *Genet. Res., Camb.* 37:227.
- J. McKenzie and P. Parsons (1972). *Oecologia* 10:373.
- J. McKenzie and P. Parsons (1974). *Genetics* 77:385.
- J. McKenzie and S. McKechnie (1978). *Nature (London)* 272:75.
- J. McKinley-McKee and D. Moss (1965). *Biochem. J.* 94:16p.
- J. McKinley-McKee and D. Moss (1965). *Biochem. J.* 96:583.
- J. Malpica and J. Vassalo (1980). *Nature (London)* 286:407.
- M. Mares-Guia (1968). *Arch. Bioch. Bioph.* 127:317.
- E. Margoliash /

E. Margoliash (1963). Proc. Natl. Aca. Sci. U.S.A. 50:672.

E. Margoliash and E. Smith (1965). In: "Evolving Genes and Proteins", p 221, ed. V. Bryson and H. Vogel, Academic Press, New York.

E. Margoliash, S. Ferguson-Miller, D. Brautigan and A. Chiavano (1976). In: "Structure - Function Relationships of Proteins", pp 143-165, ed. R. Markham and R. Horne, Elsevier/North Holland: New York.

G. Maroni (1978). Biochem. Genet. 16:509.

E. Mendez and C-Y. Lai (1975). Anal. Biochem. 65:281.

G. Miller and J. Golder (1950). Arch. Biochem. 29:420.

G. Miller (1959). Anal. Chem. 31:964.

c. P. Milstein and C. Milstein (1968). Biochem. J. 109:93

H. Michl (1951). Monatsh. Chem. 82:489.

D. Moras, K. Olsen, M. Salveson, M. Buehner, A. Ford and M. Rossmann (1975). J. Biol. Chem. 250:9137.

P. Morgan (1975). Heredity 34:124.

D. Morris and J. McKinley-McKee (1972). Eur. J. Biochem. 29:515.

T. Mukai, L. Mettler and S. Chigusa (1971).

Proc. Natl. Aca. Sci. U.S.A. 68:1065.

H. Müller (1950). Am. J. Hum. Genet. 2:111.

J. Oakeshott (1976). Genet. Res., Camb. 26:265.

J. Oakeshott (1977). Aust. J. Biol. Sci. 30:259.

J. Oakeshott, /

- J. Oakeshott, J. Gibson, D. Anderson and A. Champ (1980).
Aust. J. Biol. Sci. 33:105.
- J. O'Brien and R. McIntyre (1969). Am. Natur. 103:97.
- R. Offord (1966). Nature (London) 211:591.
- T. Ohta and M. Kimura (1971). J. Mol. Evol. 1:18.
- T. Ohta and M. Kimura (1971). Nature (London) 233:118.
- T. Ohta (1972). J. Mol. Evol. 1:305.
- T. Ohta and M. Kimura (1973). Genet. Res., Camb. 22:201.
- T. Ohta and M. Kimura (1975). Am. Natur. 109:137.
- K. Okuda and N. Tagigawa (1970). BBA 220:141.
- S. Pipkin and N. Hewitt (1972). J. Heredity 63:267.
- S. Pipkin, C. Rhodes and N. Williams (1973). Genetics 74:3213.
- S. Pipkin, C. Rhodes and N. Williams (1973). J. Heredity 64:181.
- S. Pipkin, E. Franklin-Springer, S. Law and S. Lubera (1976).
J. Heredity 67:258.
- A. Place, D. Powers and W. Sofer (1980).
Federation Proceedings Supplement 39:1640.
- J. Powell (1971). Science 174:1035.
- S. Prakash, R. Lewontin and J. Hubby (1969). Genetics 61:841.
- C. Prosser /

C. Prosser (1967). In: "Molecular Mechanisms of Temperature Adaptation", Am. Assoc. Adv. Sci. Public, Washington D.C.

B. Rasmuson, L. Nilson, M. Rasmuson and E. Zeppezauer (1966).
Hereditas 56:313.

A. Riggs (1976). Federation Proceedings 35:2115.

M. Rossmann, M. Anderson, M. Buehner, A. Ford, M. Hackert, P. Lentz,
A. McPherson, R. Schweitz and I. Smiley (1971).
Cold Spring Harb. Symp. Quant. Biol. 36:179.

M. Rossmann, M. Adams, M. Buehner, A. Ford, M. Hackert, A. Liljas,
S. Rao, L. Banaszak, E. Hill, D. Tsernoglou and L. Webbs (1973).
J. Mol. Biol. 76:533.

M. Rossmann, A. Liljas, C. Bränden and L. Banaszak (1975).
In: "The Enzymes", Vol XI, pp 62-103, ed. P. Boyer, Academic Press,
New York.

Y. Rustum, E. Massaro and E. Barnard (1971). Biochemistry 10:3509.

A. Ryle, F. Sanger, L. Smith and R. Kitai (1955). Biochem. J. 60:541.

B. Sampsell (1977). Biochem. Genet. 15:971.

F. Sanger and J. Thompson (1953). Biochem. J. 53:353.

A. Scheffer (1973). Dr. Nat. Sci. Thesis, University of Groningen,
Netherlands.

M. Schwartz, /

- M. Schwartz, L. Gerase, J. O'Donnel and W. Sofer (1975).
In: International Conference on Isoenzymes, 3rd, Yale University,
Vol I, Molecular Structure, Academic Press, New York.
- M. Schwartz and H. Jöörnvall (1976). Eur. J. Biochem. 68:159.
- M. Schwartz and W. Sofer (1976). Genetics 83:125.
- M. Schwartz, J. O'Donnel and W. Sofer (1979).
Arch. Bioch. Bioph. 194:365.
- G. Schwert, B. Miller and R. Peanasky (1967).
J. Biol. Chem. 242:3245.
- R. Selander and S. Yang (1969). Genetics 63:653.
- R. Selander and S. Yang (1970). Science 169:179.
- R. Singh, J. Hubby and R. Lewontin (1974).
Proc. Natl. Aca. Sci. U.S.A. 71:1808.
- R. Singh, J. Hubby and L. Throckmorton (1975). Genetics 80:637.
- R. Singh, R. Lewontin and A. Felton (1976). Genetics 84:609.
- L. Smith, M. Nava and E. Margoliash (1973). In: "Oxidase and
Related Redox Systems, Vol 2, pp 629-638, ed. T. King, H. Mason and
M. Morrison, University Park Press, Baltimore.
- W. Sofer and H. Ursprung (1968). J. Biol. Chem. 243:3110.
- W. Sofer and M. Hatkoff (1972). Genetics 72:545.
- C. Tanford (1962). Adv. Prot. Chem. 17:69.
- C. Tanford /

- C. Tanford and R. Roxby (1972). *Biochemistry* 11:2192.
- D. Thatcher (1977). *Biochem. J.* 163:317.
- D. Thatcher and R. Camfield (1977). *Bioch. Soc. Trans.* 5:271.
- D. Thatcher (1980). *Biochem. J.* 187:975.
- D. Thatcher and L. Sawyer (1980). *Biochem. J.* 187:885.
- D. Thatcher and B. Hodson (1981). *Biochem. J.* 197:105.
- D. Thatcher and A. Retzios (1981).
Protides Biol. Fluids Proc. Colloq. 28:157.
- D. Thatcher and R. Sheikh (1981). *Biochem. J.* 197:111.
- H. Theorell and McKinley-McKee (1961). *Acta. Chem. Scand.* 15:1811.
- J. Thompson and T. Kaiser (1977). *Heredity* 38:191.
- H. Ursprung and J. Leone (1965). *J. Exptl. Zool.* 160:147.
- H. Ursprung and L. Carlin (1968). *Ann. N.Y. Aca. Sci.* 151:456.
- H. Ursprung, W. Sofer and N. Burroughs (1970).
Wilhem Roux Archiv. 164:201.
- W. van Delden, A. Kamping and A. van Dijk (1975). *Experientia* 31:418.
- W. van Delden, A. Boerena and A. Kamping (1978). *Genetics* 90:161.
- W. van Delden and A. Kamping (1979). *Genet. Res., Camb.* 33:15.
- G. Vigue /

- G. Vigue and F. Johnson (1973). Biochem. Genet. 9:213.
- G. Vigue and W. Sofer (1976). Biochem. Genet. 14:127.
- R. Voekler, R. Mettler and T. Mukai (1978). Genetics 88:515.
- S. G. Walley and J. Watson (1953). Biochem. J. 57:529.
- M. Walsh (1973). In: "Alcoholism", ed. P. Bourne and R. Fox, Academic Press, New York.
- K. Wang (1967). BBA 133:369.
- R. Ward and P. Herbert (1972). Nature (London) 236:243.
- R. Ward (1974). Biochem. Genet. 12:449.
- R. Ward (1975). Genet. Res., Camb. 26:81.
- J. Whitaker, D. Yates, J. Holbrook and A. Gutfreund (1974). Biochem. J. 139:677.
- J. White, M. Hackert, M. Buehner, M. Adams, G. Ford, D. Lentz, I. Smiley, S. Steindel and M. Rossmann (1976). J. Mol. Biol. 102:759.
- A. Wilks, J. Gibson, J. Oakeshott and G. Chambers (1980). Aust. J. Biol. Sci. 33:575.
- G. Winter (1977). Ph.D. Thesis, University of Cambridge, U.K.
- C. Woenckhaus, J. Berghäuser and G. Pfeiderer (1969). Hoppe-Seyler's Z. Physiol. Chem. 350:473.
- K. Woods /

K. Woods and K. Wang (1967). BBA 133:369.

J. Wooton (1974). Nature (London) 252:542.

E. Zuckerkandl and L. Pauling (1962). In: "Horizons in Biochemistry", pp 189-225, ed. M. Kasha and B. Pullman, Academic Press, New York.

APPENDIX I: Purification, amino acid analysis and sequencing
of the soluble peptides resulting from the tryptic
digestion of an ADH-F enzyme preparation derived
from a population (Kaduna; Nigeria) of *Drosophila*
melanogaster

An effort was undertaken recently by investigators to measure the level of silent substitutions, that is, substitutions that result in no net charge alterations and which theoretically should comprise 2/3 of the total.

Thermostability tests, gel filtration and electrophoresis in a series of gels of variable acrylamide concentrations were some of the techniques employed (Singh et al, 1976; Coyne, 1976). Claims have been made that through the use of one or a combination of these methods the amount of observed variation in several loci of *Drosophila* species and other insect populations has been increased dramatically (Singh et al, 1975; Singh et al 1976; Coyne, 1976; Johnson, 1977; Coyne et al, 1978). Such observations will tend to support the neutralist theory as formulated by King and Ohta (1975). In most cases though the new variants recognised by such techniques have not been structurally investigated and therefore final confirmation is missing. Similar techniques employed by Sampsell (1977), Kreitman (1980) and McKay (1981) failed to provide any evidence for appreciable amounts of silent substitutions in ADH in populations of *Drosophila melanogaster*.

Flies from a Kaduna (Nigeria) population of *Drosophila melanogaster* (grown as outlined in section 2.2) were homogenised and ADH-F was purified according to the methods described in Chapter 2. ADH-S present in the homogenate was excluded by sticking to the hydroxylapatite column, while ADH-F was retrieved on passing through.

Tryptic digestion, soluble peptide purification and characterisation were performed along the lines employed for the ADH-F' structural investigation. The larger amounts used in tryptic digestion ($\approx 4 \mu$ moles) allowed the retrieval of both T1 (the blocked N-terminal tryptic peptide) and T4 peptides that were not isolated from the ADH-F tryptic digest. The arginine content of the T4 peptide was overestimated (Table A.1) due to reasons mentioned in section 2.3. Unexpectedly, a new UV-fluorescent peptide appeared (Table A.1, Fig. A.1) close to the position usually occupied by the T14b peptide, present in ADH-S digests. Analysis and sequencing showed that this peptide (termed T14c) resulted from a "pseudotryptic" cleavage on peptide T14 after the Phe-193 residue. It was identical with the chymotryptic peptide T14c2 identified by Thatcher (1980). The amino acid analysis of the T12 peptide showed lower valine content than expected but results were affected by the low recovery (usual for the peptide) and the short (24 h-long) acid hydrolysis. Among the larger peptides T7 was purified in this case by paper methods. Tyrosine detected on polyamide thin layer chromatography sheets during sequencing was not shown in the amino acid analysis results either because of decomposition or because of its conversion to chlorotyrosine.

Amino acid analysis also underestimated the threonine content of this peptide, but such a result was the consequence of the large size of the sample, the number of residues of this amino acid and the limitations of the Rank-Hilger Chromaspek analyser. The resulting peak was too large to be accurately measured by the analyser.

In conclusion, the use of HVPE, BAWP chromatography, amino acid analysis and sequencing revealed no evidence of silent substitutions in the part of the sequence covered by the soluble tryptic peptides (approximately 60% of the total). The results, though negative, confirm the conclusions of Sampsell (1977), Kreitman (1980) and McKay (1981). However, it must be noted that protein chemistry techniques would not have detected a silent substitution (or substitutions) accounting for less than 10% of the purified preparation.

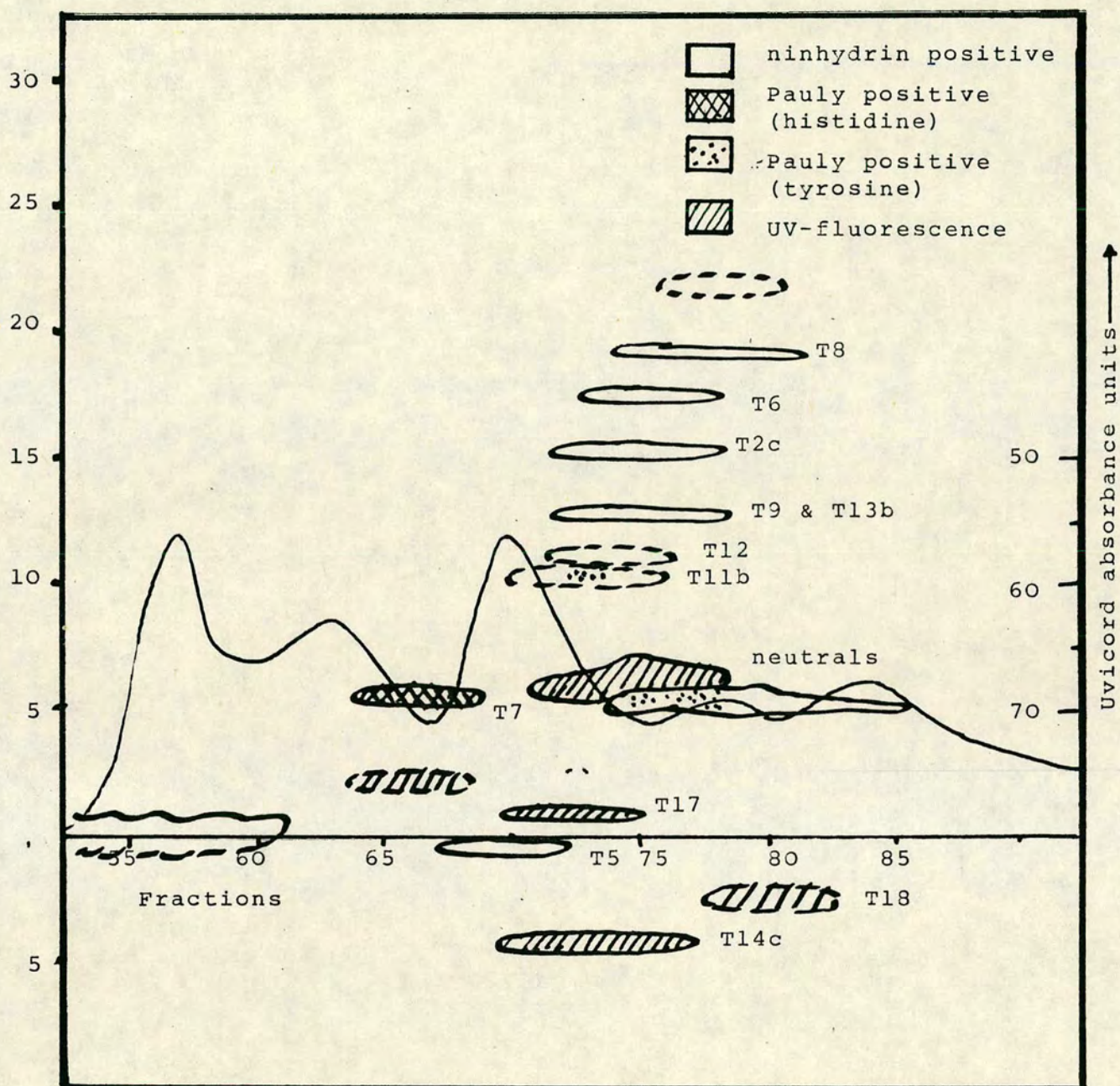


Fig. A.1. : HVPE at pH 6.5 of the tryptic digest of oxidized *Drosophila* ADH from a Kaduna population, after fractionization by Sephadex G-25 SF gel filtration.

Table A.1 : Quantitative amino acid analysis of soluble tryptic peptides of Kaduna population ADH-F

Amino acid peptide		Asx	Thr	Ser	Glx	Pro	Gly	Ala	Val	Ile	Leu	Tyr	Phe	His	Lys	Arg
T8	(a)										1.94				1.09	
	(b)										2				1	
T6	(a)	1.06				0.99		0.83		1.18					1.11	
	(b)	1				1		1		1					1	
T2c	(a)				0.83						1.83				1.04	1.30
	(b)				1						2				1	1
T13b	(a)	1.03	1.68			0.94	1.02		0.98	1.01						1.00
	(b)	1	2			1	1		1	1						1
T9	(a)		1.01		1.06			1.13		0.77	0.96		0.91		0.94	
	(b)		1		1			1		1	1		1		1	
T12	(a)	1.22	1.56	1.24				2.59	1.25		1.37		0.97		0.84	
	(b)	1	1	2				3	2		1		1		1	
T11b	(a)	1.06	1.00	0.88	1.12	0.83	1.23	1.21	2.04	1.02		1.60			0.94	
	(b)	1	1	1	1	1	1	1	2	1		2			1	
T1	(a)	1.08	1.89	0.82							1.00		0.96		0.95	
	(b)	1	2	1							1		1		1	
T3	(a)	1.03									0.98				0.98	
	(b)	1									1				1	

CONTINUED/

Table A.1 : (continued)

Amino acid peptide		Asx	Thr	Ser	Glx	Pro	Gly	Ala	Val	Ile	Leu	Tyr	Phe	His	Lys	Arg
T2b	(a)				1.12						1.78				1.10	
	(b)				1						2				1	
T16	(a)	2.30			1.43		1.18	1.61		1.61	1.48				1.00	
	(b)	2			2		1	2		2	1				1	
T13a	(a)		1.80			1.10	1.10	2.05	1.02	0.94	0.85	0.89				
	(b)		1			1	1	2	1	1	1	1				
T4	(a)	2.13							0.82	0.87	1.86					1.92*
	(b)	2							1	1	2					1
T17	(a)	1.15	2.02		2.47		1.04	0.85		0.81	2.57				0.91	
	(b)	1	2		2		1	1		1	3				1	
T5	(a)	1.05			2.20	0.98		3.00		1.87	0.83				0.93	
	(b)	1			2	1		3		2	1				1	
T7	(a)	1.08	3.23 [†]		1.08	1.98		1.24	2.46	1.32			0.70		0.91	
	(b)	1	5		1	2		1	3	1		1	1		1	
T14c	(a)	1.57		0.84	3.04	1.23		1.22	1.90		0.98				0.95	
	(b)	2		1	3	1		1	2		1				1	

* Overestimate due to large overlapping ammonia peak.

† Underestimate due to the size of the peak that exceeded the range of the instrument.

APPENDIX II: Presentation of the measurements and results of kinetic experiments on ADH-F, ADH-S and ADH-F' (Chapter 4) as Lineweaver - Burk (1934) plots. The intercepts are the computer provided solutions (section 2.4.2.(e)).

